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Review

Volatile fatty acids production in ruminants and the role of monocarboxylate transporters: A review

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Monocarboxylates commonly referred to as short-chain fatty acids (SCFAs) are metabolized to different extents by the epithelium of the gastrointestinal tract. They are absorbed along different segments of the gastrointestinal tract and constitute a significant amount of energy in ruminants. Monocarboxylates play a central role in cellular metabolism and metabolic communication between tissues. Essential to these roles is their rapid transport across the plasma membrane, which is catalyzed by a recently identified family of proton-linked monocarboxylate transporters (MCTs). Monocarboxylate transporter-1 and 4 have been shown to interact specifically with OX-47 (CD147), a member of the immunoglobulin superfamily with a single transmembrane helix. This interaction appears to assist MCT expression at the cell surface. Despite the importance of short-chain fatty acids in being the main energy source in ruminant animals, the mechanism of SCFAs transport and absorption is still not fully studied. The aim of this review is to critically discuss short-chain fatty acids production and the functional role of monocarboxylate transporters in relation to the transport and absorption of these nutrients along the gastrointestinal tract of ruminants. Two major functions of monocarboxylate transporter proteins, namely the facilitation of the absorption of SCFAs in the gastrointestinal tract and the regulation of cell pH in skeletal muscles, are clearly very important for physiological homeostasis, animal welfare and productivity.

Key words: Ruminants, monocarboxylates, monocarboxylate transporters, CD147.

INTRODUCTION

Most mammals are herbivorous and are involved in the consumption of plant material high in structural carbohydrates. Consequently, these groups of animals have evolved a close symbiotic relationship with the microorganisms that reside in their gut which are engaged in the digestion of highly fibrous plant material for the host. The ruminant animal has evolved a specially adapted digestive system to enable, for the best part, a relatively efficient breakdown of feedstuffs and is divided into four different compartments, the reticulum, rumen, omasum and abomasum. The rumen is the main site of microbial digestion and is best described as a large fermentation vat which contains a complex variety of different microorganisms which act synergistically to break down feed for the host animal. After extensive fermentation by the resident microbes, the products of fermentation, mainly organic volatile fatty acids (VFAs) and microbial protein then become available to the host. Gruzdev et al. (2001) established a high proportions of rumen volatile fatty acids (acetic, butyric and propionic) in young Friesian bulls 2 hours post-feeding with mixed rations of low-degraded neutral detergent fiber in dry matter content; and these volatile fatty acids peaked for 4hrs in the rumen after feeding. Conditions in the rumen are strictly anaerobic, although small trace amounts of oxygen may be found, particularly in close proximity to the rumen wall and in ruminal gas. In the gastrointestinal tract, monocarboxylate transporters are important in the transport of short-chain fatty acids and lactate (Ritzhaupt et al., 1998a,b; Muller et al., 2002; Koho et al., 2005).

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Abbreviations: SCFAs, Short-chain fatty acids; **MCTs,** monocarboxylate transporters; **VFAs,** volatile fatty acids; **RT-PCR,** reverse transcription-polymerase chain reaction.

The aim of this review is to critically discuss volatile fatty acids or short-chain fatty acids (SCFAs) production and the functional role of monocarboxylate transporters in relation to the transport and absorption of short-chain fatty acids along the gastrointestinal tract of ruminants.

SHORT CHAIN FATTY ACIDS PRODUCTION AND THEIR FUNCTIONS IN DIFFERENT SEGMENTS OF THE GASTROINTESTINAL TRACT OF RUMINANTS

Short chain fatty acids such as acetic, propionic, butyric, isobutyric, valeric, isovaleric, 2-methylbutyric, hexanoic and heptanoic acid, are produced in several parts of the gastrointestinal tract by microbial fermentation of dietary fibre. They constitute weak acids, but because the pH of the gastrointestinal tract, with the exception of the stomach, is nearly neutral, 90–99% of SCFAs are present as anions rather than as free acids. In all mammals examined, acetate is the main SCFA produced. Propionate and butyrate are also present in large concentrations, although their amounts can vary considerably with diet. The molar ratios of acetate to propionate to butyrate in mammals vary from approximately 7.5:15:10 to 40:40:20 (Bergman, 1990). An estimated, 60 – 70% of the energy of the epithelium of the colon is derived from SCFAs, particularly from butyrate (Scheppach et al., 1992). Butyrate also possessed other important functions in the intestinal epithelium, such as prevention of certain types of colitis (Scheppach, 1994), while acetate increases colonic blood flow and enhances ileal motility (Scheppach, 1994).

SCFAs or volatile fatty acids are the products of the anaerobic microbial fermentation of complex carbohydrates in the forestomach and large intestine. Acetate, propionate and butyrate, the predominant SCFAs, are readily absorbed and assimilated as a nutrient source by the ruminant (Bergman, 1990). Ruminants depend on SCFAs for up to 80% of their maintenance energy requirements (Bergman, 1990). Caecal SCFAs provide, on average, 8.6% of metabolizable energy intake in bovines (Siciliano-Jones and Murphy, 1989). Caecal and colonic fermentation accounts for 8.6 - 16.8% of total SCFAs production in ruminants (Ulyatt et al., 1975). In addition to their involvement as the major source of energy, the SCFAs also serve as building blocks for milk synthesis; acetate is a necessary component in the formation of milk fat, while propionate is used for glucose production, which is needed for synthesis of milk sugar (lactose). In ruminants, propionate is also the major substrate of hepatic gluconeogenesis (Herdt, 1988). Thus, effective absorption of SCFAs from the forestomach and large intestine is essential for these species. In addition, the three main SCFAs, acetate, propionate and butyrate stimulate sodium and fluid absorption in the colon and exert proliferative effects on colonocytes (Scheppach, 1994). Lactate is mostly absorbed in the small intestine (Argenzio and Southworth, 1974). In the gut, the abundance of the monocarboxylate transporter (MCT) protein in the colonic luminal membrane declines during transition from normal differentiation and proliferation in the colonic mucosa (Ritzhaupt et al., 1998). It has been established in the colon *in vivo* that a reduction in MCT1 expression, and hence butyrate transport, can lead to a reduction in intracellular butyrate levels (Daly et al., 2005). Decreased butyrate concentration results in dysfunction in the regulation of genes associated with colonic tissue homeostasis and disease prevention (Daly et al., 2005), thus, leading to impairment of animal welfare.

MONOCARBOXYLATE TRANSPORTERS

Monocarboxylic acid transporters (MCTs) members of SLC16A family are proton-linked transporters that play a crucial role in cellular metabolism. To date, fourteen MCT related sequences have been identified in mammals through sequence homology; however, only seven isoforms have been functionally characterized (Halestrap and Meredith, 2004; Murakami et al., 2005). These isoforms differ in terms of tissue distribution, substrate specificities and affinities with only four isoforms (MCT1-4) characterized as proton-dependent monocarboxylate transporters (Halestrap and Meredith, 2004; Bonen et al., 2006).

In all species studied, efflux of lactate and protons is facilitated mainly by MCTs, which transport a proton and a lactate anion through the cell membrane (Juel, 1998; Halestrap and Price, 1999). Among monocarboxylates, the transport of lactate is quantitatively the most important, but MCTs also transport other monocarboxylates such as butyrate, acetate and propionate, which are important especially in the gastrointestinal tract (Halestrap and Price, 1999). Of the 14 different MCT isoforms (MCT1-MCT14) identified so far, MCT1, MCT2 and MCT4 are the best characterized and known to transport lactate (Halestrap and Meredith, 2004). These three isoforms have different species- and tissue-specific distributions (Halestrap and Price, 1999). TAT1 (MCT10) has been shown to transport aromatic amino acids (Halestrap and Meredith, 2004), and MCT8 to be an active and specific thyroid hormone transporter (Friesema et al., 2003). In addition, MCTs in the epithelium of the small intestine, colon and blood-brain barrier provide routes for many carboxylated pharmaceutical agents (Enerson and Drewes, 2003). All MCTs are membrane proteins, with 12 transmembrane regions and cytoplasmic N- and C- terminal ends (Poole et al., 1996; Halestrap and Price, 1999). They transport a monocarboxylate anion and a proton together through the cell membrane according to the electrochemical gradients of substrates. MCT-1 and 4 need a chaperone protein, CD147 (also known as basigin, EMMPRIN, HT7 or OX-47), in muscle (Halestrap and Price 1999; Juel and Halestrap 1999), red blood cells (RBCs) (Koho et al., 2002) and the intestine

(Buyse et al., 2002). The suggested model of the topology of CD147 and MCT1 in the plasma membrane is a dimmer of CD147 associated with two MCT1 molecules such that the C-terminus of CD147 in the cytosol is close to the C-terminus of its partner CD147 and to the C- and N- termini of an associated MCT1 molecule (Wilson et al., 2002).

FUNCTIONAL ROLE OF MONOCARBOXYLATE TRANSPORTERS IN DIFFERENT PARTS OF THE GASTROINTESTINAL TRACT OF RUMINANTS

In the gastrointestinal tract, monocarboxylate transporters are important in the transport of short-chain fatty acids and lactate (Ritzhaupt et al., 1998a, b; Muller et al., 2002, Koho et al., 2005). The two major functions of monocarboxylate transporter proteins, namely the facilitation of the absorption of SCFAs in the gastro-intestinal tract and the regulation of cell pH in skeletal muscles, can thus be assumed to be very important for physiological homeostasis and also for animal welfare.

The transport of short-chain monocarboxylates across the plasma membrane in most cells is largely dependent on a family of specific MCTs. Kirat et al. (2005, 2006) established that MCT1 is expressed along the gastrointestinal tract of preruminant calves, and adult sheep, respectively. Thus, it can be suggested that MCT1 may possibly play a vital role in the transport of SCFAs across the ruminant gastrointestinal tract. Kirat et al. (2006) using reverse transcription-polymerase chain reaction (RT-PCR) established that MCT1 mRNA was highly expressed in bovine large intestinal mucosa. Both immunohistochemistry and confocal laser microscopy verified that the MCT1 protein was abundant in the surface epithelium of the large intestine, and the amount decreased from the opening of the crypt to its base. In the immunopositive cells, MCT1 was primarily localized in the basolateral membranes of epithelium lining the large intestine. MCT1 is an obligatory symporter that carries a dissociated proton-monocarboxylate pair with each transport cycle (Halestrap and Meredith, 2004). Also, Kirat et al. (2006) using RT-PCR revealed the presence of mRNA encoding for MCT1 in all regions of the caprine gastrointestinal tract. Quantitative western blot analysis also showed that the level of MCT1 protein was in the order of rumen ≥ reticulum > omasum > caecum > proximal colon > distal colon > abomasum > small intestine. Amongst the stratified squamous epithelial cells of the forestomach, MCT1 was predominantly expressed on the cell boundaries of the stratum basale and stratum spinosum. Double-immunofluorescence confocal laser-scanning microscopy confirmed the co-localization of MCT1 with its ancillary protein, CD147 in the caprine gastrointestinal tract (Kirat et al., 2006). Kirat et al. (2007) established the precise cellular localization of MCT4, along with its co-existence with its chaperone, CD147 in the ruminant gastrointestinal tract. Using quantitative western blot analysis, they demonstrated that the abundance of MCT4 protein was in the order of forestomach > large intestine > abomasum ≥ small intestine. Immunohistochemistry and immunofluo-rescence confocal laser microscopy also showed that MCT4 in the forestomach was confined to the cell membranes of strata corneum and granulosum, while diffuse cytoplasmic staining for MCT4 was visualized in strata spinosum and basale. In the epithelium cells lining the abomasum, MCT4 immunoreactive positivities were predominantly localized on the basolateral membranes. In the small intestine, MCT4 was localized at the brush borders and the basolateral membranes of the epithelial cells lining the villi, however, it was mostly found on the apical membranes of the crypt cells.

In the large intestine, the immunoreactivity for MCT4 differed between the surface epithelium and the crypts; in the surface epithelium, MCT4 was mainly localized at the apical membranes, whereas in the crypts it was predominantly expressed on the basolateral membranes of the lining epithelial cells. MCT4 was remarkably co-existed with CD147 along the bovine gastrointestinal tract.

CONCLUSION

Short-chain fatty acids (acetate, propionate and butyrate) are the end products of anaerobic microbial fermentation of carbohydrates in ruminant gastrointestinal tract. They represent the most predominant anions in the ruminant forestomach and large intestine. They are readily absorbed into the blood stream and transported to body tissues where they are used for hepatic gluconeogenesis, lipogenesis in peripheral tissues and milk synthesis. The transport of short-chain monocarboxylates across the plasma membrane in most cells is largely dependent on monocarboxylate transporter family. There is a potential functional collaboration between MCT1 and MCT4; this may provide new insights into the mechanisms that mediate the transport of short-chain fatty acids and other monocarboxylates in the different segments of the ruminant gastrointestinal tract.

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Review

Treatment of wastewater from rubber industry in Malaysia

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Presently, Malaysia is the third largest rubber producer in the world, whereby the rubber industry is an economically and socially significant industry. Rubber industry consumes large volumes of water, uses chemicals and other utilities and produces enormous amounts of wastes and effluent. Discharge of untreated rubber effluent to waterways resulted in water pollution that affected the human health. With a new global trend towards a sustainable development, the industry needs to focus on cleaner production technology, waste minimization, utilization of waste, resource recovery and recycling of water. The present work aims at highlighting various technologies that currently have been used for treatment of rubber effluent in Malaysia. The work introduces the basis of these processes including their benefits and also problems. It also adheres to the future trends of rubber effluent treatment in Malaysia by reviewing various treatment technologies for natural rubber industry implemented by Thailand, the world largest rubber producer. These new and effective effluent treatment methods would minimize environmental pollution of rubber industry and bring it to become sustainable and environmental friendly.

Key words: Rubber industry, effluent, waste management, Malaysia.

INTRODUCTION

Natural rubber is an elastic hydrocarbon polymer that is originally derived from a milky colloidal suspension, or latex of *Hevea brasiliensis*. The purified form of rubber which can also be produced synthetically is chemical polyisoprene. Natural rubber is extensively used in various applications and products (Sun, 2004). Today, 70 -80% of produced raw rubber in the world supply in south-east Asia, comes mainly from Thailand, Malaysia and Indonesia (Lonholdt and Andersen, 2005; Xiaofei, 2008). Main export of Malaysian products includes electronic equipment, petroleum and petroleum products, palm oil, wood and wood products, rubber and textile (Usa, 2007). Rubber plays an important role in Malaysian's economy and about 30% of foreign exchange revenue derived from this crop (Shacklady, 1983; Hutagalung, 2003; Anitha et al., 2007). However, some changes in land use were raised in the mid-80s and large areas of rubber field were converted for industrial, commercial and residential uses (Vijayaraghavan, 2008a, b). More tendencies were also done towards the plantation of lucrative crops likes oil palm and cocoa (Choo et al., 2003). Due to this, a gradual decrease occurred in raw natural rubber production. Therefore, It caused a decline to Malaysian's status from first to third and ranked it after Thailand and Indonesia (Vijayaraghavan, 2008a). The natural rubber statistic (2008) for Malaysia and Thailand is shown in

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Abbreviations: EQA, Environmental quality act; **UASB,** anaerobic sludge blanket reactor; **SRR,** sulphate reduction reactor; **PNSB,** purple non sulphur photosynthetic bacteria; **CW,** constructed wetland; **VF,** vertical flow; **SSF,** subsurface flow; **ORP,** oxidation-reduction potential; **BAS,** batch activated sludge; **DOE,** Department of Environment; **RRIM,** Rubber Research Institute of Malaysia; **BOD,** biological oxygen demand; **HRT,** hydraulic retention times; **COD,** chemical oxygen demand; **TDS,** total dissolved solids.

Table 1. Rubber industry in Thailand and Malaysia (Hutagalung, 2003; Kantachote and Innuwat, 2004; Puetpaiboon et al., 2005; Chaiprapat and Sdoodee, 2007; Department of Statistics, Malaysia, 2008; Association of Natural Rubber Producing Countries, 2009).

Factor	Thailand	Malaysia
Area under rubber plantation (million hectares)	2	1.3
Natural rubber production in year (million tons)	3.09	1.072
Global supply of natural rubber (percent)	33.5	10.7
Number of rubber factories	700	357

Table 2. Characteristics of process effluents from rubber processing (Chua and Garces, 1992; Guha, 1995; Bacon, 1995; Hutagalung, 2003; Choo et al., 2003; Tekasakul and Tekasakul, 2006; Chaiprapat and Sdoodee, 2007; Rungruang and Babel, 2008; Vijayaraghavan et al., 2008a).

Parameter	Typical range
pH	3.7 - 5.5
Biological oxygen demand	1500 - 7000
Chemical oxygen demand	3500 - 14000
Suspended solids	200 - 700
Total nitrogen	200 - 1800
Sulphate	500 - 2000

All units are mg/l, except pH.

Table 1 (Hutagalung, 2003; Kantachote and Innuwat, 2004; Puetpaiboon et al., 2005; Chaiprapat and Sdoodee, 2007; Department of Statistics Malaysia, 2008; Association of Natural Rubber Producing Countries, 2009).

Since the production of rubber products from natural rubber needs large amount of water for its operation, the rapid growth has produced large quantities of effluent from this processing (Leong et al., 2003; Rungruang and Babel, 2008). This effluent includes wash water, small amounts of uncoagulated latex and serum with small quantities of protein, carbohydrates, lipids, carotenoids and salts. The characteristics of rubber wastewater are presented in Table 2 (Chua and Garces, 1992; Guha, 1995; Bacon, 1995; Hutagalung, 2003; Choo et al., 2003; Tekasakul and Tekasakul, 2006; Chaiprapat and Sdoodee, 2007; Rungruang and Babel, 2008; Vijayaraghavan et al., 2008a).

According to Department of environment (DOE, 2000), the biggest sources of industrial water pollution in Malaysia are food and beverage producers, chemical based industries, textiles, paper, palm oil and rubber processing (Shirkie and Ching, 1983; Usa, 2007; Iyagba et al., 2008). 14.1% of the 2292 industries in Malaysia which identified as major sources of water pollutants are rubber producing industries (DOE, 1991). The main sources of rubber wastewater in Malaysia are skim, latex serum, uncoagulated latex and washings from the various processing stages (Abdul Rani, 1995). In average, 20 tons of rubber and 410 thousand litres of effluent per day is produced by rubber factory. Some environmental pollution has been reported due to daily discharge of about 80 million litres of untreated rubber effluent to near streams and rivers in Malaysia (Hutagalung, 2003; Tekasakul and Tekasakul, 2006; Rungruang and Babel, 2008).

The Malaysian Government instituted a set of environmental regulations that are included in the Environmental Quality Act (EQA) of 1974, and its subsequent amendments in 1985 and 1996 (Yassin, 2008).

In Malaysia, the legislative approach in water quality management and raw natural rubber industry was established in 1977-1978 (Chua and Garces, 1992; Daud, 2005; Usa, 2007). The effluent standards set by DOE, are given in the Table 3 (Choo et al., 2003).

Without proper treatment, the discharge of wastewater from rubber processing industry to the environment may cause serious and prolong consequences. Therefore, suitable technologies must be used for treating this wastewater (Yassin, 2008). Rubber industries in Malaysia have implemented treatment facilities that consistent with regulations. Biological methods especially aerobic, anaerobic and facultative ponds are widely used for treatment of rubber wastewater in Malaysia (Usa, 2007). These systems are inexpensive and have a high efficiency for organic load reduction, but are appropriate for areas that land is available. Therefore, mechanical treatments such as anaerobic filter beds, rotating biodiscs and aerated lagoons are currently being used where land is limited (Chua and Garces, 1992; Kolmetz et al., 2003; Kantachote and Innuwat, 2004). As a result, the net organic

Table 3. Maximum standards for effluent discharge into wastewater for the rubber industry (Choo et al., 2003).

Parameter	Concentration (mg/l), 1984 and later
Biological oxygen demand	100
Chemical oxygen demand	400
Total solids	1000
Suspended solids	150
Ammonical nitrogen	300
Total nitrogen	300

Source: Environmental Protection Act 1996.

load of Malaysian's rubber effluents has been reduced by 80% in 1980-1985 (Chua and Garces, 1992; Daud, 2005). Most of rubber treatment plants now comply with the standards, but high pollutants concentration remains from rubber, because of improper discharges of a significant number of smallholdings and lack of adequate maintenance of their effluent treatment systems (Chua and Garces, 1992; Abdul Rani, 1995; Cleary and Chuan, 1999). In order to carryout organic pollution reduction, developing of improved purification systems for rubber effluents is essential (Guha, 1995). Also persistent attention and novel treatment methods will require for protection of Malaysia's rivers (Shirkie and Ching, 1983).

Another serious threat of rubber wastewater towards environmental protection is high concentration of nitrogen in this effluent. It contributes to undesirable eutrophication, economic loss, methemoglobinemia in infants, increases oxygen and chemical demands and affects the paddy field. Application of sulphuric acid in the coagulation of skim latex results in production of high level of sulphate in the effluent of rubber processing factories. The high levels of hydrogen sulphide (H_2S) will be liberated to the environment and cause malodour problems. The free H_2S also inhibits the digestion process, which gives lower organics removal efficiency. The odours are detectable even at extremely low concentrations and make water unpalatable for several hundred miles downstream from the rubber processing factories (Rungruang and Babel, 2008). Therefore, treatment of rubber wastewater using effective methods for overcoming to these problems is needed. Several processes have been developed for treatment of this wastewater in Malaysia. Several processes have been developed for treatment of this wastewater in Malaysia which will be discussed in details in this paper

CONVENTIONAL/CURRENT RUBBER WASTEWATER TREATMENT METHODS

Malaysian rubber wastewater treatment process was provided in 1995 (Lonholdt and Andersen, 2005). Various Wastewater treatment systems have been adopted by Malaysian rubber industry (Table 4). It has been found that the biological processes such as pond technology and aerobic and anaerobic methods are the most commonly used treatment systems. The existing biological method was also incorporated with sulphate reduction system and precipitation in order to further improve the efficiency of rubber wastewater treatment. Azolla and water hyacinth as a biological method and also deammoniation system are other alternative techniques that currently have been used for treatment of rubber effluent in Malaysia (Table 5).

BIOLOGICAL METHOD OF RUBBER WASTEWATER TREATMENT

Pond technology

Pond technology is a widely used method for treatment of effluents from the primary rubber industry in Malaysia. There are more than 500 palm oil and rubber factories in Malaysia which almost all have installed some type of primary wastewater treatment system like ponds (Usa, 2007). The pond systems usually include high loaded anaerobic ponds and low loaded aerobic ponds. Before the pond treatment system, there is a latex trap and a neutralization stage as a pretreatment step. If the ponds are effectively designed and optimally operated, it can remove over 95% biological oxygen demand (BOD) from rubber wastewater (Ahmad et al., 1980). Effluent with BOD levels of 50 - 100 mg/l can normally be achieved by methods mentioned above (Lonholdt and Andersen, 2005).

Moreover, pond technology is an alternative for rubber wastewater treatment in other countries like Thailand. Rakkoed and co-workers (1999) have used two kinds of waste treatment ponds (waste stabilization ponds and attached-growth waste stabilization ponds) as to compare the efficiency of nitrogen removal from concentrated latex wastewater. Working dimensions of each pond was 0.4 m^3 width, 1.2 m^3 depth and 0.6 m^3 length. During the first, second and last experiment run, hydraulic retention times (HRT) of 40 and 20 days, 40 days with 50% recirculating of effluent and 4 days was conducted. The results revealed that total Kjeldahl nitrogen (TKN), ammonia nitrogen (NH_3-N) and BOD_5 removal efficiencies in attached-growth waste stabilization ponds were higher than in control ponds. This is because of an increase in biomass on media in the pond water. In other hand, it has been reported that there is a treatment system in each co-operative

Table 4. Various rubber wastewater treatment systems and their efficiency

Treatment	Description	Initial COD (mg/L)	Initial BOD (mg/L)	Initial TKN (mg/L)	Initial sulphide (mg/L)	COD removal efficiency (%)	BOD removal efficiency (%)	TKN removal efficiency (%)	Sulphate removal efficiency (%)	SS removal efficiency (%)	Reference
Conventional/Current technologies											
Anaerobic filter.	Packed with aquarium media with dimension of 30 cm *100 cm; OLR = 11.8 $gCODL^{-1}$ day^{-1} and HRT = 10 days.	18219	12750	-	-	92	-	-	-	-	Anotai et al. (2007)
Up-flow anaerobic sludge blanket (UASB).	Steel cylinder shape with dimension of 600 m^3 *250 m^3, consists of a waste water distributor, a lid for scraping sludge, dry rubber content and a gas-solids separator.	6100	-	315	-	80	-	80	90	-	Taechapatarakul (2008)
Biological method incorporated with sulphate reduction system (purple non-sulphur photosynthetic bacteria).	Optimum growth in latex rubber sheet wastewater with 0.50% ammonium sulphate and 1 mg/l nicotinic acid in a pure culture and or a mixed culture.	7328	4967	-	-	90	90	-	92 - 96	-	Kantachote et al. (2005)

operative rubber sheet factories in Thailand that it is minimally maintained, and not being functioned based on the design. Actually, there are two models of the treatment systems that consist of four ponds with different sizes according to the available land of factory. Due to high maintenance and energy costs of surface aerator, all of these factories had stopped using it. This makes to low quality of the effluent from these systems which unable to meet the industrial effluent standards in Thailand (Chaiprapat and Sdoodee, 2007). A series of ponds were also used for treatment of 4.58 m^3/ton of wastewater from smoked sheet rubber factories in southeast Thailand. In this study, HRT 90-120 days were used for reducing the nitrogen of rubber wastewater. The results showed that the number and size of ponds were not adequate for treatment of wastewater. Therefore, it caused to serious water pollution and unpleasant odour to the neighbouring villagers (Puetpaiboon et al., 2005; Taechapatarakul, 2008). In general, settling ponds have few drawbacks which it takes sixty days. That means a great amount of water has to be kept over time for treatment. This method needs a large area of land, operating cost and time (Clay, 2004; Shamsudin et al., 2006).

Aerobic and anaerobic treatment

Biological treatment of rubber wastewater is a method, which gives the greatest reduction of BOD (Rungruang and Babel, 2008). Aerobic and anaerobic treatment is the most common biological method used in Malaysia for treating rubber wastewater as it is an inexpensive treatment with high efficiency (Kolmetz et al., 2003; Kantachote and Innuwat, 2004). In some rubber factories where land area is limited, aeration systems are used as an alternative of settling ponds. Conventional treatment systems

Table 4. Continued.

Treatment	Description	Initial COD (mg/L)	Initial BOD (mg/L)	Initial TKN (mg/L)	Initial sulphide (mg/L)	COD removal efficiency (%)	BOD removal efficiency (%)	TKN removal efficiency (%)	Sulphate removal efficiency (%)	SS removal efficiency (%)	reference
Advanced technologies											
Natural process.	Consists of VF followed by SSF with nut grass (*Cyperus rotundus* Linn) plantation.	5750 - 18303	780 - 9625	102 - 157.6	85 - 460	99	99	97.8	93.6	-	Puetpaiboon et al. (2005)
Electrochemical methods.	Electrolytic reactor, anode and catode with dimension of 300*, 450, 270*, 60, 270 and *50 mm; thickness = 0.8 mm and electrolysis period = 90 min.	2000 - 6000	1000 - 3500	250 - 700	250 - 400	99.9	98.8	-	-	-	(Vijayaraghavan et al., 2008a)
Ozonation followed by batch activated sludge process.	ozone dosage = 66.44 mg O_3/ L O_2 of; pH = 9.0 and contact time = 30 min.	999 - 1160	350 - 398	165 - 199	1642 - 2045	91.49	95.79	67.95	74.68	-	Rungruang and Babel (2008)
Combined physical, chemical and biological methods (gas injection technique).	-	-	-	-	-	67	77	51	95	-	(Yusoff et al., 2004)

are well set up using aeration systems with sludge recycling to increase the microbial concentrations in the aeration tanks (Shamsudin et al., 2006). It can remove over 95% of BOD from the rubber wastewater (Ahmad et al., 1980). This system can overcome malodour and are suitable for factories with inadequate land area. However, the existing system was not effective in removing nitrogen because of an insufficient supply of dissolved oxygen for nitrification (Nordin, 1990).

In 1971, the Rubber Research Institute of Malaysia (RRIM) used the biological processes for the treatment of rubber factory effluent. This system consists of improved anaerobic and aerobic processes. Coconut fibre as a new medium was selected for rubber wastes treatment. The availability, high specific surface area, high water holding capacity and a balanced C/N/P ratio of coconut fibre are favourable characteristics for biofiltration applications (Karim and Vaishya, 1999; Suwardin et al., 1999; Gabriel et al., 2007). Since, waste oil palm fibres have a shorter retention time and narrower space; it is also possible to get the high efficiency in wastewater treatment using these media (Suwardin et al., 1999).

Several types of enclosed anaerobic digesters have also been evaluated for treatment of rubber effluent in lab scale. Hollow cylindrical clay, poly-urethane foam and rubberised coir were chosen as a packing material in these digesters (Ahmad, 1983; Zaid, 1988, 1992) for attaching the microbial growth in anaerobic filter as a packed bed reactor, used form of inert static medium. It

Table 5. Other rubber wastewater treatment methods.

Treatment method	Description	Result	Reference
Azolla as a biological treatment.	Using the tiny floating fern *Azolla* for treating of wastewater from sugar refineries and rubber processing plants in Malaysia.	Extraction of compounds containing nitrogen.	MacKinnon et al. (1996).
Water hyacinth as a biological treatment.	Plants such as water hyacinth used for treatment of Malaysian rubber sewage and recovery of dissolved nutrients.	A reduction of 99 and 80% in BOD and COD. Decline the suspended solids of treated wastewater to the less than 50 mg/l using combined system. More efficiency of combined algae-water hyacinth system for removal of nitrogen as compared to the normal high rate algal pond.	Kulatillake and Yapa (1986); Gamage and Yapa (2001); Bich et al. (1999) .
Deammoniation system.	Removal of ammonia from skim latex using a slatted-plate system and a new deammoniation system, known as POTORRIM that was developed by RRIM.	Overcoming to some problems encountered with slatted-plate system provides more efficient deammonification and effective cleaning using POTORRIM.	Nordin (1993).

was revealed that the filter can accept loadings of up to 8 kg COD/m^3/day. The results also showed that only 50% of chemical oxygen demand (COD) was removed that could possibility be due to packing used (Ahmad, 1983). At the same loading rate, higher COD removal (70%) was achieved with polyurethane foam and hollow cylindrical clay (Zaid, 1988). Moreover, clogging may be a main problem with these packed bed systems and the influents need to be filtered prior to treatment. This problem has led to the development of anaerobic sludge blanket reactor (UASB) (Ahmad et al., 1986).

Taechapatarakul (2008) used UASB reactor which is in a steel cylinder shape with the dimensions of 600 and 250 m^3. It worked 8-24 h a day and consists of a waste-water distributor, a lid for scraping sludge, dry rubber content and also a gas-solids separator. It has been found that the UASB are able to carryout agglomeration of granular sludge forming a stable blanket in the reactor. Fast breakdown of organic materials can be achieved due to the ability of granular sludge for retention of high solids in the reactor. UASB system is a close system that can control the fault smells which comes from applied oxidation and stabilization ponds. Moreover, this system consumes low electricity energy that can help saving a lot of money. The main problem of the UASB is difficulty in developing the granular sludge blanket and maintaining its stability. However, this system was used in many industries but it still never approved that it could work appropriately with natural rubber wastewater (Taechapatarakul, 2008).

Combined aerobic and anaerobic digestion has been used for biogas production from rubber wastewater in Batang Kali, Selangor, Malaysia. This plant produced about 8000 m^3 of biogas per day that will be used as a fuel in the boilers for the production of steam. These technologies are commercialised in the Philippines, Hong Kong and Singapore (http://www.cogen3.net/fsdp-rubber malay.html). Moreover, the 32 manufacturing plants in Thailand produced 254 million m^3 per year of methane from solid waste and wastewater (Priwan, 2008).

On the other hand, aerobic and anaerobic treatment methods are not acceptable by government and industry due to problems about control of microoganisms such as introduction of unknown foreign species, genetic mutation, insufficient scientific evidence, high cost of technology and others (Tan, 2007).

BIOLOGICAL METHOD INCORPORATED WITH SULPHATE REDUCTION SYSTEM

Low cost operation, high removal efficiency and also producing the biogas as a useful energy sources are some advantages of anaerobic wastewater treatment system (Kantachote et al., 2008). However, this treatment results in the formation of H_2S due to consumption of sulphate instead of oxygen by sulphate-reducing bacteria. H_2S is toxic and increases the smell of putrid eggs. The gas also causes a big problem in biogas producing systems (Kantachote and Innuwat, 2004). As a result, sulphide could inhibit the activity of methane producing bacteria due to its toxicity. It also revealed that the high amount of sulphide reduced the COD removal. Therefore sulphide elimination is an important stage for this kind of

wastewater before a biogas production step (Kantachote et al., 2008).

Sulphate reduction reactor (SRR) has been used for treatment of sulphate rich rubber wastewater from concentrated latex and skim crape. The SRR is needed for reduction of sulphate concentration in wastewater before biogas production by UASB. However, the produced biogas does not have a good quality due to its high amount of H_2S. Therefore, the biogas was burnt to remove the very toxic and corrosive H_2S gas.

Hence, converting the sulphide to sulphur by partial oxidation is needed. It is realized that levels of sulphide oxidation are dependent on oxygen concentration. Additionally, bacteria with ability of oxidizing reduced sulphur compounds can be used for removal of H_2S from treated wastewater or gaseous systems. Thus, selection of a microbe that can grow at room temperatures and neutral pH with ability of oxidizing sulphide to sulphur in wastewater is important (Taechapatarakul, 2008; Kantachote et al., 2008).

Identification of bacteria which can grow in the concentrated latex wastewater was studied by Choorit et al. (2003). In this work, the efficiency of the isolated strains for organic content reducing of concentrated latex effluent was evaluated. The purple non-sulphur photosynthetic bacteria which were isolated from a concentrated latex effluent were cultured in a wastewater without any supplementation. After 40 h of cultivation, 34% of COD was decreased by *Rubrivivax gelatinosus* and *Thiobacillus* sp. (Choorit et al., 2003).

Thiobacillus sp. meanwhile is extensively used worldwide for removal of both organic and inorganic sulphur compounds in wastewater. *Thiobacillus* sp. can reduce inorganic sulphur compounds as an energy source and therefore is used for removing sulphide from wastewater. Four kinds of *Thiobacillus* sp. were isolated from domestic and rubber wastewaters in Thailand by Kantachote and Innuwat (2004). All isolates could grow in pH of 2.0 - 7.0 (optimum 6.5), temperature of 25 - 45°C (optimum 30 - 35°C) under both aerobic and anaerobic conditions. The results showed that the highest COD removal (54%) can be obtained by *Thiobacillus* sp. WI1 which cultivated in rubber sheet wastewater for 14 days. However, it does not show the good ability for BOD reduction and it declined by only 33%. Against, the efficiency of strain WI4 for BOD and COD removal was 83 and 46% (Kantachote and Innuwat, 2004).

Kantachote et al. (2005) also isolated some purple non-sulphur photosynthetic bacteria (PNSB) from rubber sheet wastewater in Thailand. Isolate DK6 as a kind of *Rhodopseudomonas* shows the best potential for effluent treatment since it can grow well under microaerobic-light conditions and a mixed culture. It has been found that the mixture of 0.50% ammonium sulphate and 1mg/l nicotinic acid with latex rubber sheet wastewater makes the optimum growth of DK6. Using these conditions and either under a pure or a mixed culture, it can reduce the COD and BOD of wastewater to 90%.

Therefore, it can be concluded that using the bacteria strains for rubber wastewater treatment is an environmental friendly method and ecologically balanced. This technology is also applicable in other organic waste disposal and has good potential in Malaysia and other Asian countries (Kamaruddin, 2007).

BIOLOGICAL METHOD INCORPORATED WITH PRECIPITATION

One of the parameter that can affect the efficiency of biological treatment processes is the presence of heavy metals such as zinc in wastewater. Adsorption, membrane separation and precipitation are some examples of effective technologies that have been used for removal of heavy metals from wastewater (Agamuthu, 2004; Wright and Nebel, 2007). Currently, simple and inexpensive method such as precipitation by hydroxide is the more common approach that are used in Malaysia. However, this method is not suitable for highly organic polluted rubber wastewater due to zinc-organic ligand complexes production. Therefore, reduction of organic matter that includes heavy metals from wastewater is required. It has been found that some kinds of microorganisms in anaerobic and aerobic processes can be used for this purpose. Microbial flocculation under aerobic conditions can be avoided by high amount of total dissolved solids (TDS) in rubber wastewater. In order to meet the effluent standards, rubber factory have to use some tertiary treatment such as coagulation and filtration processes to remove the excess solids. This significantly increases their treatment and disposal costs. Therefore, reduction of the TDS to a level that does not inhibit or interfere with aerobic microbial aggregation is required. Another method for removal of heavy metal is sulphide precipitation.

In this process, use neutral pH which is also suitable for microbial growth (Eckenfelder, 1999). In addition to, the removal efficiency of sulphide precipitation is usually better than the hydroxide treatment under a low dissolved solid condition. Therefore, sulphide precipitation is a more promising option than the recent technology. In other hand, adjustment of optimal dosage in hydroxide precipitation system is much easier than sulphide method especially under frequent fluctuation of zinc concentration. In fact, high amount of sulphide can makes malodour and also excessive residual sulphide whilst inadequate dose of precipitant can results in an effluent with high amount of zinc. Therefore, study about the effect of important parameters on sulphide addition control system which is easy and cheap are needed. Chemical and biological processes without any pH adjustment were used for treatment of acidic latex wastewater with high amount of zinc. Sulphide and hydroxide precipitation increased the total dissolved solids of treated effluent by 1.1 and 2.8 times, respectively.

92% of TDS was removed by anaerobic filter in more than 11.8 $gCODL^{-1}$ day^{-1} of organic loading rate. For the activated sludge process, average removal efficiencies for COD and BOD were 96.6 and 99.4%. This combined system was verified to be an effective method for purification of rubber thread wastewater (Anotai et al., 2007).

Another most cost effective system for zinc removal from the wastewater in Malaysia is using a mixture of 800 mg/l of sodium sulphide and 5 mg/l of polyelectrolyte LT27, respectively. The optimum settling time and flocculation time were 60 and 20 min. The best results can be obtained in a speed of 20 rpm in a 110 mm diameter reactor. The approximate cost of this system is RM1.04/m^3 (US \$0.26/m^3) of wastewater discharged (Subbiah et al., 2004).

In a study by Kolmetz et al. (2003), the efficiency of an expanded bed biofilm reactor in the treatment of wastewaters contaminated with heavy metals has been investigated for rubber product manufacturing industry. Some advantages of biofilm systems are ability to retain relatively high biomass concentrations that results in shorter liquid retention times, better performance stability and higher volumetric removal rates. In the study, it has been found that the process could achieve 60 to 90% removal of Zinc. In addition, the efficiencies of an expanded bed biofilm reactor and a sequencing batch biofilm reactor for heavy metal adsorption were studied using Zn and Cu containing wastewaters. The results showed that heavy metal adsorption by these reactors are 50 - 95% (Mohamad, 2007).

ADVANCED RUBBER WASTEWATER TREATMENT METHODS

Currently, several effective methods have been used for treatment of rubber wastewater in Thailand which makes natural rubber industry more environmentally friendly and economically viable. In response to problems associated with rubber wastewater and its effect on the Malaysian's environment, further research can be performed to develop the novel methods for treatment of this wastewater. As follow some inventive technologies that were used in Thailand and Malaysia will be described. The best novel methods also are shown in Table 4.

Natural process

A constructed wetland is an artificial marsh or swamp that includes substrate, vegetation and biological organisms contained within a physical configuration. Suitability designed and operated wetlands have considerable potential for low-cost, efficient and self maintaining wastewater treatment systems. This system has demonstrated capability to remove nutrients, suspended solids, organic compounds, pathogens and metallic ions and to increase oxygen and pH levels in wastewater. In comparison to conventional systems, lagoons or land application flow, wetlands waste treatment systems require fewer amounts of capital and operating costs, minimal operator training and land area (Hammer, 1989).

Puetpaiboon et al. (2005) studied the possibility of treat wastewater from a rubber sheet factory in Thailand using the pilot-scale experiment constructed wetland (CW). This system consisted of vertical flow constructed wetlands (VF) followed by subsurface flow constructed wetlands (SSF) with nut grass (*Cyperus rotundus* Linn.) plantation. The tested COD loadings in this experiment were 500, 750, 1000 and 1250 kg COD/ha.d. The results showed that the best removal efficiency of BOD5, COD, SS and TKN were 99, 99, 93.6 and 97.8%, respectively, using VF followed by SSF with nut grass (*C. rotundus* Linn.) plantation at 750 kg COD/ha.d.

Biological method

One of the extensively used methods for removal of dissolved and colloidal organics in wastewater is the activated sludge process. This system changes the dissolved and colloidal organic contaminants to a biological sludge which can be removed by settling. After a primary settling basin, usually use an activated sludge process as a secondary treatment (Cheremisinoff, 1998). Chevakidagarn and Ratanachai (2004) and Chevakidagarn (2006) surveyed two rubber treatment plants, which are single-stage activated sludge process in Songkhla, Thailand. They reported that the suspended solids removal capacities were low (from 78 to 87%). The treatment plants often had bulking and rising sludge problems because of insufficient oxygen concentrations. Thus, it is recommended that an appropriate oxygen control system to be included in this system for the rubber industry to ensure sufficient oxygen is provided. This will assist in compliance with the effluent standards. Chevakidagarn et al. (2006) also upgraded the operation of conventional activated sludge treatment plants to save aeration energy and at the same time to provide better utilization of existing plant capacity for nutrient removal without major financial investment. The first stage of the experiments was to observe the possibility of using oxidation-reduction potential (ORP) for aeration control in treatment plant fed with the wastewater from the latex rubber industry. The results proved that the ORP was greatly affected by the change in air supply. However, it was also affected by the fluctuation of wastewater temperature, which contributed to the bulking sludge problem.

In another study, Chevakidagarn (2005) used surrogate parameters for rapid monitoring of contaminant removed for activated sludge treatment plant for rubber and seafood industries in Southern Thailand. UV absorbency

at various wavelengths was used in this study as surrogate parameters, for predicting the removal capacity of each plant. The results showed that UV absorbency at 220 nm can be used as a parameter to predict nitrate-nitrogen concentrations which less than 15 mg/l. Also, it was found that 550 and 260 nm are suitable wavelengths for predicting of suspended solids concentration and COD.

In generally, aerated lagoon or activated sludge can reduce fault smell of rubber wastewater but have a high consumption of electricity and relatively high investment and operation costs (Puetpaiboon et al., 2005; Taechapatarakul, 2008).

Chemical methods

Electrochemical methods: Longer hydraulic retention time is needed for treatment of rubber wastewater by conventional biological methods and sometimes exposure to failures if shock loaded. Recently, much more attention has been drawn to electrochemical method for treatment of wastewater due to the cost, ease of control and the increased efficiencies provided by the use of new electrode material and compact biopolar electrochemical reactor (Sequeira, 1994; Rajeshwar and Ibanez, 1997; Vijayaraghavan et al., 2008b). The electrochemical treatment methods are preferred as the less required of hydraulic retention time.These systems are not successful because of variation in wastewater strength or due to the existence of toxic material. Generation of chlorine or hypochlorous acid is the best alternative method for performing the electrolytic treatment. Therefore, there is a considerable interest for developing a new method of wastewater treatment based on *in situ* hypochlorous acid generation as a kind of electrochemical method (Vijayaraghavan et al., 2008a, b). An electrolytic cell including of graphite as anode and stainless sheets as cathode was providing the hypochlorous acid. For organic destruction of the rubber wastewater used, the produced hypochlorous acid acts as an oxidizing agent. The results showed the efficiency of 99.9 and 98.8% for COD and BOD removal. Moreover, it has been indicated that pH 7.3; TOC 45 mg/l; residual total chlorine 136 mg/l; turbidity 17 NTU and temperature 54EC can be obtained by this system for an influent with the initial pH 4.5; current density of 74.5mA/cm^2; sodium chloride content 3% and electrolysis period of 90 min, respectively. At the same condition and up to 2% of sodium chloride concentration, 95.7 and 88.6% of COD and BOD removal, pH 7; TOC 90 mg/l; residual total chlorine 122 mg/l; turbidity 26 NTU and temperature 60EC can be achieved (Vijayaraghavan et al., 2008a).

Ozonation

Some of the non-biodegradable materials and also ammonia nitrogen of natural rubber wastewater cannot be completely removed using biological process. Therefore, the organic contents of effluent are above those of the standard limits. It is generally approved that ozonation transforms refractory or poorly degradable organic materials into by-product with smaller molecular size. Moreover, it has been found that ammonia can be converted to nitrate by ozonation which usually makes biodegradability.

Rungruang and Babel (2008) studied the efficiency of batch activated sludge process (BAS) with or without ozonation for rubber wastewater treatment at Chonburi, Thailand. The effects of different contact times (0 - 90 min) and ozone dosages (37.20, 56.90 and 66.44 mg O_3/ L O_2) at various pH (7.4, 9.0 and 11.0) on wastewater treatment were studied. As a result, it was found that 66.44 mg O_3/ L O_2 of ozone dosage; pH of 9.0 and 30 min of contact time are the optimum conditions for reduction of pollutants. It has been found that the combination of BAS and ozonation makes higher removal efficiency for all parameters compared to another system (Rungruang and Babel, 2008; Spellman, 2003; Qasim, 1998).

Combined physical, chemical and biological methods

The combined processes were found to be very effective in treatment of rubber wastewater in Malaysia and Thailand. Iyagba et al. (2008) reported that for removing the high levels of ammonia nitrogen from anticoagulants which is used in treating raw rubber of Asia-Pacific Region, aeration alone systems is not efficient. Combined physical and chemical treatment systems followed by two-stage biological method was used for fibre and suspended solids removal in Malaysia. This work involved the installation of a fibre screening system, a dissolved air flotation system, an aeration basin, a secondary clarifier and a sludge de-watering system. This process could produce an effluent which is consistent with the DOE Standard A (Kolmetz et al., 2003).

Application of membrane technology that involves gas injection technique is another alternative for treatment of natural rubber effluent in Malaysia. The results showed that total permeate flux was enhanced from 8.3 to145.3% using gas sparging technique. In terms of permeate quality, 95, 67, 77, 51, and 74%, of reductions were achieved for total solids, COD, BOD, total nitrogen and NH_3-N, respectively. For the non-gas sparging system, permeate flux declined sharply with time due to the accumulation of foulant on the membrane surface. However, both conditions showed increase of total permeate flux with transmembrane pressure and feed flowrate (Yusoff et al., 2004).

55 concentrated latex factories exist in southeast Thailand which also used combination of physical and biological methods for treatment of wastewater. Trapping of rubber as a physical process was used for removal of suspended rubber. The biological treatment that was utilized in 22 factories was

stabilization pond which includes anaerobic, facultative and aeration ponds. Anaerobic ponds with aerated lagoon were installed in 24 factories and in the 5 of them, activated carbon processes were utilized. UASB and land application were another methods that used by 1 and 3 factories. All of these technologies were not suitable for reduction of COD, BOD, magnesium and sulphate from concentrated latex wastewater but are capable for hardly removing nitrogen and phosphorous (Furumai et al., 2007).

CONCLUSION

Recently, several processes have been used for treatment of rubber wastewater in Malaysia. Advantages and disadvantages of these methods rely on simplicity, flexibility and effectiveness of the operation, cost, technical problems and maintenance. Therefore, more economical alternative technologies for the treatment of wastewater from rubber factories are required. Conventional treatment options have been favoured in the past for treatment of rubber effluent but biological treatment especially aerobic, anaerobic and facultative ponds are becoming increasingly popular since they are inexpensive and have a high performance for organic load reduction. Moreover, novel systems for management of agro-industrial wastes have been developed to overcome the problems of conventional systems. However, these advanced methods should be calibrated based on rubber effluent characteristics in Malaysia and, at the same time, the efficiency of these methods has to be improved. Generally, production of good effluent quality from rubber wastewater treatment can be accelerated by these innovative techniques.

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Full Length Research Paper

Application of SRAP in the genetic diversity of *Tricholoma matsutake* in northeastern China

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Tricholoma matsutake **is an ectomycorrhizal (ECM) fungus that produces economically important mushrooms. The study firstly applied SRAP technique into genetic diversity of *T. matsutake.* A total of 129 strains from 13 geographical locations in northeastern China, were amplified by using selected 12 primer pairs. The polymorphic band number amplified by each primer pair ranged from 7 to 13. In total 154 bands were observed, of which 118 were polymorphic (76.62%). Abundant genetic variation was detected within individual populations. Dongning maintained a higher genetic diversity while Hunchun was lower. The analyses found a significant positive correlation between genetic distance and geographical distance and no correlation between genetic distance and altitudinal differences among populations. Based on the UPGMA cluster diagram, the 13 populations may be divided with the genetic distance of 0.035 into three groups.**

Key words: Genetic diversity, *Tricholoma matsutake*, fungi, sequence related amplified polymorphism (SRAP).

INTRODUCTION

The ectomycorrhizal fungus *Tricholoma matsutake* is one of the most delicious and valuable edible mushrooms in Asia, especially in Japan, Korea, and China (Hall et al., 2003). Through northeastern China, *T. matsutake* grows widely in coniferous trees, especially *Pinus densiflora* forests, and sometimes it can also be found in *P. thunbergii, P. pumila*. While in southwestern China, it grows in broad-leaved forests mainly consisting of *Castanopsis* spp. and *Quercus* spp. (Wang et al., 1997). In the past century, because of deforestation and infestation by the pinewood nematode (*Bursaphelenchus xylophilus*), the host plant populations of *T. matsutake, P. densiflora* declined rapidly in Japan (Wang and Hall, 2004). As a result, the annual harvest of *T. matsutake* in Japan has been much lower than it used to be in the early 20th century. Because artificial cultivation has not been developed for any of the matsutake mushrooms, including *T. matsutake*, to satisfy its domestic demand, Japan imports about 3000 tons of *T. matsutake* annually, mostly from Pacific North America, Korea and China. As an important production region, Heilongjiang and Jilin in northeastern China accounts for 39% of the total amount of the exported *T. matsutake* of China.

Although *T. matsutake* mushrooms have a high commercial value and a very important role in non-timber forest products, very little is known about the relationships among geographical populations, either in Japan or elsewhere. A study has examined the relationship between genetic distance and geographical distances in *T. matsutake* (Chapela and Garbelotto, 2004). In this study, seven strains of *T. matsutake*, three from China and one each from Korea, Japan, France, and Morocco, were examined. A significant positive correlation was found between their genetic dissimilarity based on genotypes identified using amplified fragment length polymorphisms (AFLP) and their geographical distances. Sha et al. (2007) sequenced the internal transcribed spacer (ITS) and the intergenic spacer (IGS) regions of the rDNA, and found no polymorphism among 56 fruiting bodies collected from 13 counties in Yunnan.

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Abbreviations: ECM, Ectomycorrhizal; **SRAP,** sequence-related amplified polymorphism; **AFLP,** amplified fragment length polymorphisms; **ITS,** internal transcribed spacer; **IGS,** intergenic spacer; **PCR,** polymerase chain reaction; **FST,** mean level of genetic differentiation.

Table 1. Geographic distributions of samples in northeastern China.

Sampling site	Latitude (North)	Longitude (East)	Altitude (m)	Sample size (N)	Year
Hailin	44.65	129.15	421	3	2005
Dongjingcheng	43.58	129.34	453	15	2007
Jidong	45.12	131.27	358	12	2006
Muling	44.97	130.65	534	6	2008
Dongning	43.24	130.59	296	16	2007
Wangqing	43.32	129.78	689	9	2008
Baijin	42.48	129.37	515	10	2007
Fuyu	41.23	127.54	752	9	2006
Fuxing	42.16	129.07	810	13	2005
Longmen	42.73	129.18	712	11	2006
Hunchun	42.86	130.34	605	10	2005
Changxing	43.17	128.95	783	7	2007
Shimen	43.28	128.78	653	8	2006
Total sample	41.23-45.12	127.54-131.27	296-810	129	2005-2008

High polymorphism could be detected, however, in strains from Japan using a specific PCR primer. Sequence-related amplified polymorphism (SRAP) is a new PCR-based marker, firstly raised by Li and Quiros, (2001). SRAP was similar to RAPD, but it was a preferential random amplification of coding regions in genome. SRAP had been applied extensively in genetic diversity analysis (Ferriol et al., 2003) and comparative genetics (Lin et al., 2004) of different species. However, up to now, the SRAP molecular marker had not been used to examine the patterns of genetic variation of *T. matsutake* from within and between populations. In this work, PCR–SRAP marker was adopted to study the genetic variation present in 129 strains from 13 geographical regions from northeastern China. The results will contribute to the conservation and sustainable utilization of *T. matsutake* of northeastern China.

MATERIALS AND METHODS

Sporocarp sampling

Sporocarps were collected during fruiting periods, from early September until the end of October of 2005 to 2008. All natural samples of *T. matsutake* were collected from forests consisting of *P. densiflora,* as the dominant species (with a frequency higher than 85%), *Quercus mongolica, Betula davurica* and shrub communites with *Rhododendron davuricum*, *Philadelphus schrenkii*. These fruiting bodies were collected from thirteen sites of northeastern China, including five in Heilongjiang and eight in Jilin. The investigated region stretched about 200 km from east to west and about 400 km from south to north, with an altitude span from 296 m above sea level in Dongning to 810 m in Fuxing. The counties, where the sampling sites were located, are shown in Table 1. A total of 129 fruiting bodies were collected, processed and stored at -70°C. The fruiting bodies were typically distributed from about 10 m of each other to as far as about 1 km from each other. These specimens were identified based on their macro- and micro morphological characteristics and confirmed based on their sequences at the internal transcribed spacer (ITS) region of the ribosomal DNA.

DNA isolation

Crude genomic DNA was extracted from sporocarps using a modified cetyl-trimethylammonium bromide (CTAB) method (Lian et al., 2003). The final DNA was suspended in 50 ml TE buffer and stored at -20°C.

SRAP-PCR amplification

A total of 132 different combinations of primers were employed using 11 forward and 12 reverse primers (Table 2). About two thirds of them could produce clear bands, but considering the polymorphism and reproducibility, 12 combinations were chosen in the following study (Table 3). The thermal cycling profile was 5 min of pre-denaturing at 94°C, followed by five cycles of 1 min of denaturing at 94°C, 1 min of annealing at 35°C and 1 min of extension at 72°C, then 35 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, and a final extension step of 7 min at 72°C. The PCR mixture consists of 5 ng of DNA template, 1.0 µM of each primer, 200 µM of dNTP each, 2.5 mM of $MgCl_2$, 1 × PCR buffer, and 1U Taq DNA polymerase in a total volume of 25 µl. The amplification was performed in an applied biosystems 9800 and negative controls were run at each step in order to ensure the reliability of the genotype. The PCR products were separated on 6% denaturing polyacrylamide gel (acrylamide: bisacrylamide = 19:1) and SRAP bands were stained using silver sequence TM DNA staining reagents (PROMEGA).

Data analysis

Amplified bands were scored 1/0 as presence/absence of homologous bands for all samples. The resulting presence/absence data matrix was analyzed using POPGENE version 1.32 (Francis and Yang, 2000) to estimate the level of genetic diversity. The following genetic diversity parameters including the percentage of polymorphic bands (PPB), effective number of alleles (Ne), Shannon's information index (I) and Nei's gene diversity (H) were obtained at both species level and population level (Nei, 1987). To test for isolation by distance, genetic distances and geographical

Table 2. The forward and reverse SRAP primer combinations used in this study.

Forward primers (5'–3')	Reverse primers (5'–3')
Me1 TGAGTCCAAACCGGATA	Em1 GACTGCGTACGAATTAAT
Me2 TGAGTCCAAACCGGAGC	Em2 GACTGCGTACGAATTTGC
Me3 TGAGTCCAAACCGGAAT	Em3 GACTGCGTACGAATTGAC
Me4 TGAGTCCAAACCGGACC	Em4 GACTGCGTACGAATTTGA
Me5 TGAGTCCAAACCGGAAG	Em5 GACTGCGTACGAATTAAC
Me6 TGAGTCCAAACCGGTAG	Em6 GACTGCGTACGAATTGCA
Me7 TGAGTCCAAACCGGACA	Em7 GACTGCGTACGAATTATG
Me8 TGAGTCCAAACCGGTGT	Em8 GACTGCGTACGAATTAGC
Me9 TGAGTCCAAACCGGTTG	Em9 GACTGCGTACGAATTACG
Me10 TGAGTCCAAACCGGCTA	Em10 GACTGCGTACGAATTCTG
Me11 TGAGTCCAAACCGGAGG	Em11 GACTGCGTACGAATTCAG
	Em12 GACTGCGTACGAATTCCA

Table 3. The polymorphism from 12 SRAP primer combinations.

Primer combinations	Total number of bands	Number of polymorphic bands	Percentage of polymorphic loci (%)
Me1/ Em12	15	12	80.0
Me2/ Em2	12	10	83.3
Me3/ Em3	10	8	80.0
Me3/ Em5	14	9	64.3
Me3/ Em6	12	8	66.7
Me4/ Em6	11	7	63.6
Me4/ Em12	15	12	80.0
Me5/ Em12	13	11	84.6
Me7/ Em4	11	9	81.8
Me8/ Em4	13	10	76.9
Me10/ Em3	16	13	81.3
Me10/ Em12	12	9	75.0

distances among sites were analyzed with Mantel tests using GENAIEX, version 5 (Peakall and Smouse, 2001). Weir and Cockerham (1984) estimated the mean level of genetic differentiation (FST) using GENEPOP, version 3.4. The phylogenetic tree was constructed using a UPGMA method in Mega 2. Finally, analysis of molecular variance (AMOVA; Excoffier et al., 1992) was conducted to estimate the relative contributions of local and regional geographical separations to the overall samples in northeastern China.

RESULTS

Polymorphism and genetic diversity of *T. matsutake* populations

A total of 129 samples from 13 sites of northeastern China were amplified by using selected 12 different SRAP primer combinations. The band number amplified by each pair of primers ranged from (Me3/ Em3) 10 to (Me10/Em3) 16, with an average of 12.8. A total of 154 bands were observed, among which 118 were polymorphic (76.62%), ranging between 7 (Me4/Em6) and 13 (Me10/Em3) per primer combination, with an average of 9.83 bands per primer set. The percentage of polymorphism for each primer combination varied from 63.6% (Me4/Em6) to 84.6% (Me5/Em12; Table 3). The size of scored bands ranged from 200 to 2000 bp.

Genetic diversity data for 13 populations of *T. matsutake* based on SRAP markers are summarized in Table 4. The percentage of polymorphic loci (PPB) of the 13 *T. matsutake* populations ranged from 33.33% (Fuyu and Hunchun) to 66.67% (Dongning). The obtained effective number of alleles (Ne) ranged from 1.1559 (Hunchun) to 1.2487 (Dongning). Nei's gene diversity index (H) ranged from 0.1012 (Hunchun) to 0.1690 (Dongning). Shannon's information index (I) ranged from 0.1451 (Hunchun) to 0.2252 (Dongning).

Genetic variation among geographical populations

The authors' analyses identified a range of FST values

Table 4. Genetic diversity of 13 *T. matsutake* populations from northeastern China.

Geographical population	PPB (%)	Ne	H	I
Hailin	50.00	1.2107	0.1310	0.1991
Dongjingcheng	41.67	1.1832	0.1223	0.1715
Jidong	58.33	1.2244	0.1589	0.2067
Muling	41.67	1.1751	0.1168	0.1665
Dongning	66.67	1.2487	0.1690	0.2252
Wangqing	41.67	1.1858	0.1291	0.1783
Baijin	50.00	1.2067	0.1335	0.1922
Fuyu	33.33	1.1564	0.1023	0.1469
Fuxing	58.33	1.2289	0.1593	0.2032
Longmen	50.00	1.2175	0.1217	0.1919
Hunchun	33.33	1.1559	0.1012	0.1451
Changxing	41.67	1.1743	0.1139	0.1628
Shimen	50.00	1.1823	0.1123	0.1751

PPB, Percentage of polymorphic loci; Ne, the effective allele number; H, Nei's gene diversity; I, Shannon's information index.

between pairs of local populations. The lowest value (0.007) was found between Fuxing and Longmen, while the highest (0.174) was found between Jidong and Changxing (Table 5). Overall, the mean FST value was about 0.10. About 10% of the gene diversity was due to geographical separations between pairs of populations. Analysis of molecular variation analysis further revealed significant genetic variation ($p<0.01$) among and within 13 populations of *T. matsutake*. Of the total genetic variation, the main component (70.3%) is contributed to by the within population level, and the rest (29.7%) existed among populations (Table 6).

The relationship between genetic distance and geographical parameters

The results from the Mantel tests are shown in Figure 1. The test showed a significant positive correlation between genetic distance and geographical distance among the analyzed populations ($p = 0.013$). Based on the UPGMA cluster diagram, the 13 populations may be divided into three groups with the genetic distance of 0.035 (Figure 2). One group included Hailin, Wangqing, Fuxing, Fuyu, Longmen and Hunchun; another group, Baijin, and Changxing; the third group, Dongjingcheng, Jidong, Mulin and Dongning.

DISCUSSION

SRAP maker is more frequently used in the genetic study of vegetables and crops and rarely in the study of fungi. Sun et al. (2006) applied SRAP marker to analyze 31 different Ganoderma strains genetic diversity. In this study, the novel PCR-based SRAP marker had been successfully used in the research of genetic diversity of *T. matsutake.* The results indicated that SRAP marker was suitable for the molecular characterization and the investigation of phylogenic relationships in *T. matsutake* and proved that the use of SRAP approach was more efficient to examine the genetic diversity in basidiomycetes.

The authors analyzed a large number of geographical populations of the ectomycorrhizal mushroom *T. matsutake* from northeastern China. The results identified significant genetic variation within and between populations. Overall, these populations showed relatively low but significant genetic differentiation. Among the 13 populations, Dongning (PPB = 66.67%; Ne = 1.2487; H = 0.1690; I = 0.2252) maintains a higher genetic diversity than the others. In contrast, the level of polymorphism and genetic diversity of the Hunchun (PPB = 33.33%; Ne = 1.1559; H = 0.1012; I = 0.1451) was found to be lower than other populations (Table 4). In addition, the amount of differentiation varied between populations, with the level of differentiation correlated to some extent to geographical distances separating the populations, which is consistent with results from a previous study using different samples (Chapela and Garbelotto, 2004).

The analyses identified a range of FST values between pairs of local populations. The lowest value (0.007) was found between Fuxing and Longmen, while the highest (0.174) was found between Jidong and Changxing. The FST values observed between geographical populations of *T. matsutake* are similar to those reported in several basidiomycete species. For example, between regional populations of *Agaricus bisporus*, the FST values were found ranging between 0.019 and 0.076 (Xu et al., 2005). In *P. ferulae* and *P. eryngii,* 19 and 6 regional populations from Italy showed FST values of 0.45 and 0.10, respectively, for these two species (Urbanelli et al., 2003). In *T. matsutake,* Chapela and Garbelotto (2004) identified that

Table 5. Pairwise FST values between geographical populations of *T. matsutake* from northeastern China.

Pop ID	Shimen	Hailin	Dongjingcheng	Jidong	Muling	Dongning	Wangqing	Baijin	Fuyu	Fuxing	Longmen	Hunchun
Hailin	0.127											
Dongjingcheng	0.088	0.031										
Jidong	0.110	0.055	0.099									
Muling	0.101	0.052	0.048	0.042								
Dongning	0.033	0.065	0.096	0.077	0.051							
Wangqing	0.048	0.038	0.073	0.044	0.099	0.076						
Baijin	0.043	0.083	0.101	0.111	0.123	0.089	0.049					
Fuyu	0.051	0.047	0.084	0.065	0.087	0.038	0.075	0.064				
Fuxing	0.068	0.076	0.091	0.073	0.091	0.033	0.066	0.019	0.055			
Longmen	0.052	0.092	0.089	0.147	0.079	0.042	0.098	0.068	0.067	0.007		
Hunchun	0.074	0.087	0.096	0.087	0.071	0.049	0.035	0.065	0.086	0.082	0.065	
Changxing	0.086	0.105	0.096	0.174	0.065	0.098	0.031	0.086	0.075	0.009	0.011	0.073

Table 6. Analysis of molecular variation for SRAP data of *T. matsutake.*

Source of variation	d.f.	SS	MS	Est. var.	Total variation (%)	p
Among populations	12	101.763	8.156	0.663	29.7	0.010
Within populations	117	153.839	1.571	1.571	70.3	0.010
Total	129	255.602	9.727	2.234		

d.f., degree of freedom; SS, sum of squared observations; MS, mean of squared observations; Est. var., estimated variance.

among the seven strains they analyzed, they found a positive correlation between their pair wise genetic dissimilarity and geographical distances. Those seven strains were far apart from each other with a distance of thousands of kilometers. However, because of the small sample sizes (mostly only one strain from each country or geographical area), the level of genetic differentiation among geographical populations could not be assessed. At present, little is known about the genetic relationships of most ectomycorrhizal fungal populations from the 100 km ranges.

At present, in order to satisfy the demands of Japanese consumers, virtually all *T. matsutake* mushrooms are picked prematurely before the veils of the mushrooms are ruptured to release spores, which affects seriously the reproducibility of the mycelia and the spores and leads to the decreasing amount of *T. matsutake.* As a result, management plans should enforce the notion that at each site, a certain number of mushrooms must be allowed to mature and sporulate so as to allow future reproduction. Aside from better management decisions based on these observations, the authors believe that their population genetic study can also contribute to improved management strategy on sustainable resource utilizations of *T. matsutake* in northeastern China. It is well known that *T. matsutake* of northeastern China exists mainly in symbiosis with *P. densiflora*. However, the major host plant is *Castanopsis* spp. and *Quercus* spp. in southwestern China *T. matsutake*. Currently, whether or not such differences in host plants influence genetic differentiation among different *T. matsutake* populations is an open question. Further study is needed to address this

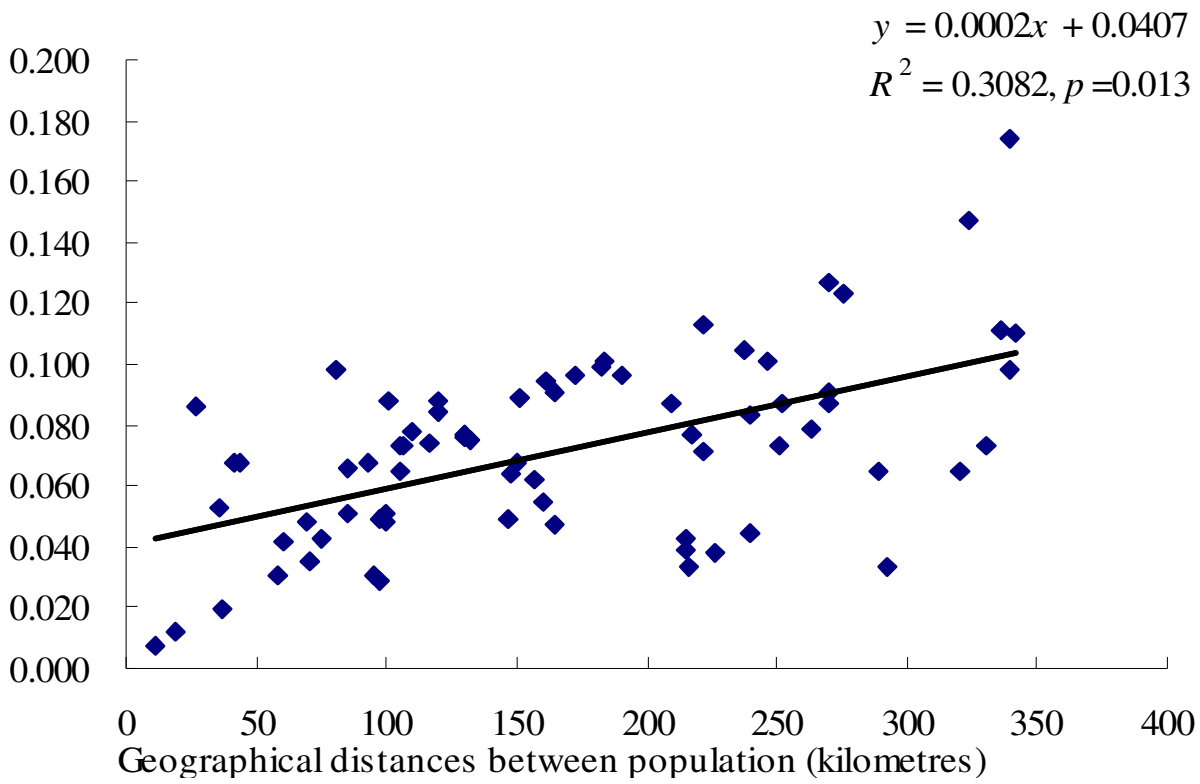

Figure 1. Results from Mantel tests between genetic differences and geographical distances among populations. The X-axis represents the geographical distance parameter and the Y-axis represents Nei's genetic distances between populations.

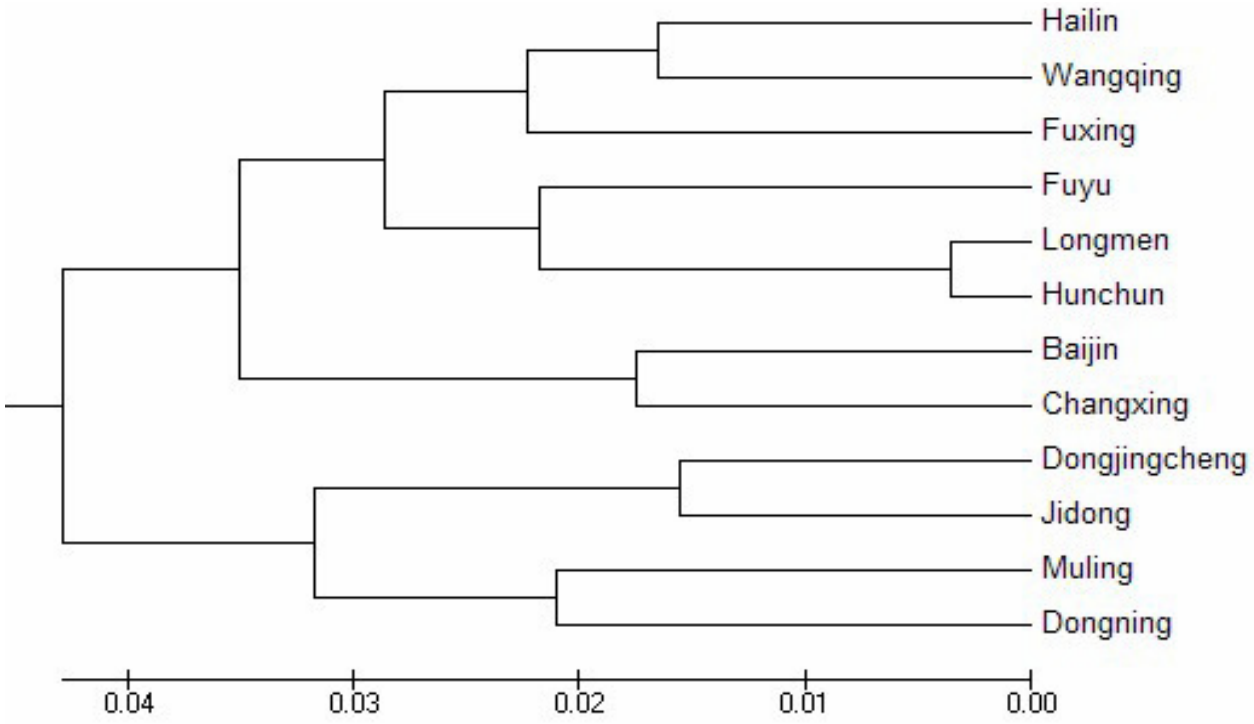

Figure 2. Genetic relationships among 13 *T. matsutake* populations.

issue.

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Full Length Research Paper

Preparation and identification of monoclonal antibodies against humanin

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To generate and characterize a monoclonal antibody (mAb) against humanin (HN), BALB/c mice were immunized with the purified pet-44a-HN in adjuvant and their splenic lymphocytes were fused with myeloma SP2/0 cells. The hybridoma cell lines were screened for HN-specific antibodies by indirect enzyme-linked immunosorbent assay (ELISA), and anti-HN mAb-producing hybridoma clones were obtained using a limiting dilution assay. The specificity and affinity of the antibodies were characterized by western blot assays and indirect ELISA. Following fusion, screening and cloning, four hybridoma clones were obtained, and the clone 5A8H3 was demonstrated to stably produce anti-HN IgG2a. Further characterization of 5A8H3 revealed that this mAb specifically recognized HN, the fusion proteins of pet-44a-HN protein and pGEMEX-1-HN, but not control (*Escherichia coli* proteins). This mAb interacted with HN at an affinity constant (Ka) of 2.0 × 10^8 M^{-1}. The HN-specific IgG2a mAb was successfully generated. It interacted with HN specifically and sensitively, providing a valuable tool for further study of the biological functions of HN.

Key words: Humanin, monoclonal antibodies, characterization.

INTRODUCTION

Humanin (HN) is a 24-amino acid peptide that was originnally found in the occipital lobe of a patient with Alzheimer's disease (AD) in 2001 (Hashimoto et al., 2001a). It can bind to its specific receptor on the cell membrane and activate the JAK/STAT3 pathway (Hashimoto et al., 2005). HN has been found to protect neurons from AD-relevant neurotoxicity (Hashimoto et al., 2001b, 2001c). It can also exhibit neuroprotective activity against toxicity by familial amyotrophic lateral sclerosis (ALS)-related mutant superoxide dismutase (Matsuoka et al., 2006). Furthermore, HN can protect lymphocytes and undifferentiated PC12 cells from serum-deprivation-promoted apoptosis (Kariya et al., 2002, 2003) and inhibit prion-peptide-induced apoptosis of cortical neurons *in vitro* (Sponne et al., 2004). However, little is known about the pattern of HN expression in rodents and humans due to the lack of specific antibody against it.

Currently, polyclonal antibodies against HN (P04) are commercially available (Tajima et al., 2002). However, because of the heterogeneous nature, polyclonal antibodies usually have low specificity. In contrast, an antigen-specific monoclonal antibody (mAb) usually has high specificity and affinity and is able to detect the antigen specifically and sensitively. Here, we report the generation and characterization of a mAb against HN. One HN-specific mAb, 5A8H3, showed high specificity and affinity. The successful generation of anti-HN mAb provides a useful tool for further investigation of the expression and function of HN.

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Abbreviations: HN, Humanin; **mAb,** monoclonal antibody; **ELISA,** enzyme-linked immunosorbent assay; **BSA,** bovine serum albumin; **IgG,** immunoglobulin G; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Materials

The recombination plasmids for pet-44a-HN and pGEMEX-1-HN were constructed in our laboratory as previously described (Luo et al., 2004). Freund's complete adjuvant (FCA) and Freund's incomeplete adjuvant (FIA) were purchased from Sigma (St. Louis, MO, USA). The HN peptide (Met-Ala-Pro-arg-G1y-Phe-Ser-Cys-Leu-Leu-Leu-Leu-Thr-Ser-Glu-Ile-Asp-Leu-Pro-Val-Lys-Arg-Arg-Ala) was synthesized by the Chinese Peptide Company (CPC, Hangzhou, China). Horseradish peroxidase-conjugated goat anti-mouse IgG (HRP-IgG) was obtained from Zhongshan Goldbridge Biotechnology (Beijing, China). RPMI-1640 medium was purchased from Hyclone (Logan, UT, USA) and fetal bovine serum (FBS) was obtained from Gibco (Grand Island, NY, USA). Polyethylene glycol 4000 (PEG4000) was purchased from Haotian Biotechnology Co. (Hangzhou, China), and the Iso-2 Mouse Monoclonal Antibody Isotyping Kit was obtained from Roche (Indianapolis, IN, USA). Mouse myeloma cell line SP2/0 was routinely maintained in our laboratory.

Mice and immunization

Female BALB/c mice (8 weeks of age) weighing about 20 g were obtained from the Laboratory Animal Center, Zhejiang University School of Medicine (Qualification No. SYXK, Zhejiang, 02004-0052). Individual mice were housed in the specific pathogen-free facility of our campus. The experimental protocols were evaluated and approved by the Animal Research Protection Committee of Zhejiang University.

Blood samples (40 µl) were collected from the ophthalmic venous plexus of individual mice prior to immunization, and the sera were used as negative controls for later experiments. Individual mice were immunized intradermally with 200 µl of the purified pet-44a-HN fusion protein (100 µg) in 50% CFA and boosted intraperitoneally with the same amount of purified pet-44a-HN fusion protein in 50% IFA twice with an interval of 10 days. Three days after the third immunization, their blood samples were collected for evaluation of antigen-specific antibody responses using indirect ELISA. After confirming the antigen-specific antibody responses, the mice were challenged intravenously with 100 µg of soluble protein antigen.

Generation of hybridoma clones

Three days after antigen challenge, splenic mononuclear cells were isolated from individual mice for generation of hybridoma clones by fusing splenic mononuclear cells with the mouse SP2/0 myeloma cells at a ratio of 10:1 in 50% (v/v) PEG 4000. The hybridoma cells were selected in HAT medium for 7 days and then maintained in HT medium for 9 – 10 days. Subsequently, their supernatants were screened for the presence of anti-HN antibodies by indirect ELISA using the HN peptide (2 µg/ml) as the coating antigen. Selected hybridoma cells were subcloned using a limiting dilution assay to obtain hybridoma clones producing antibody against HN.

Production of ascites and purification of the anti-HN MAb

Individual BALB/c mice were intraperitoneally injected with 0.5 ml mineral oil. Two weeks later, the mice were intraperitoneally injected with 10^6 hybridoma cells (0.5 ml). Ten days after injection, the generated ascites were collected by syringe and the anti-HN IgG was purified using a 1 ml protein A-agarose column (Sigma).

Characterization of the anti-HN mAb

The subclasses of anti-HN mAbs were determined using the Iso-2 Mouse Monoclonal Antibody Isotyping Kit according to the manufacturer's protocol. The affinity of anti-HN IgG was determined by indirect ELISA using different concentrations of anti-HN IgG (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} mol/L) and coating antigen (2, 4 and 8 µg/m1). The affinity constant (Ka) was calculated as previously described (Beatty et al., 1987). The titer of anti-HN IgG was detected with indirect ELISA using 2µg/ml HN peptide and HRP-IgG (diluted 1:5000) as the coating antigen and the detecting antibodies, repectively. The plate wells that had been coated with bovine serum albumin (BSA) or coated with HN peptide and probed with mouse IgG from unmanipulated BALB/c mice, were used as negative controls.

The specificity of anti-HN IgG was analyzed by western blot assays. In brief, the purified pet-44a-HN and pGEMEX-1-HN fusion protein, the synthesized HN peptide, the cell lysate of *Escherichia coli* BL21 (DE3) colonies that had been transformed with the plasmid of pet-44a-HN, pGEMEX-1-HN, or negative control pGEMEX-1, and BSA were separated by SDS-PAGE. After transferring and blocking, the HN proteins on the membranes were probed with anti-HN IgG and then detected with HRP-IgG, followed by visualization via enhanced chemiluminescence (Thermo Fisher Scientific Inc, Rockford, IL, USA) and exposure to x-ray film. The same isotype of mouse IgG was used as a negative control.

RESULTS AND DISCUSSION

To generate a mAb against HN, mice (n = 4) were immunized with the purified pet-44a-HN fusion protein in CFA and boosted with antigen in IFA, followed by challenge with soluble antigen. Their blood was collected and the anti-HN antibodies contained in the sera were identified by indirect ELISA using the synthesized HN peptide as the coating antigen. The serum titers of anti-HN IgG reached $1:10^4$ to $1:10^5$. Subsequently, their splenic mononuclear cells were isolated and fused with SP2/0 myeloma cells at a ratio of 10:1. Following selection, 25 hybridoma lines (a fusion rate of 88%) were obtained and their supernatants were screened for anti-HN IgG using indirect ELISA. Following limited dilution three times, a total of four hybridoma clones that produced HN-specific IgG were identified. Of the four hybridoma clones, the 5A8H3 clone was found to secrete the highest concentration of anti-HN IgG (30 µg/ml) in the supernatant. Importantly, after being frozen in liquid nitrogen for 6 months, this hybridoma clone continually produced high concentration of anti-HN IgG. Thus, this clone was further characterized for its antigen specificity and affinity.

Analysis of the IgG subclass of this anti-HN mAb revealed that it was IgG2a, and the titer of ascitic mAb reached $1:10^6$ (as determined by indirect ELISA). Importantly, this mAb specifically recognized the 63 KD of pet-44a-HN fusion protein and the 31 KD of pGEMEX-1-HN fusion protein, but not any of the *E. coli* proteins (no band developed in the cell lysates of *E. coli* that had not been transformed with the HN-containing plasmid) (Figure 1A). In addition, this anti-HN IgG reacted with the synthesized HN peptide (Figure 1B) but not with control BSA, suggesting that it specifically recognized HN, at

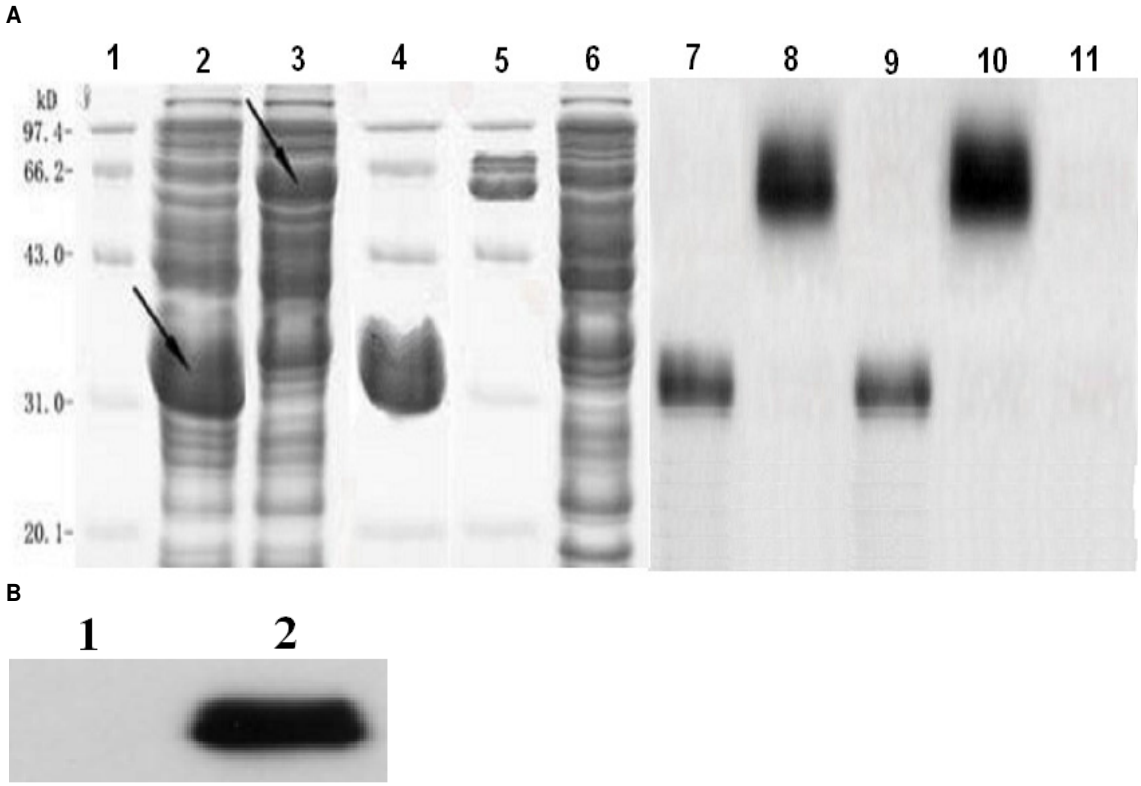

Figure 1. Western blot analysis of anti-HN IgG2a. (A): The protein markers (lane 1), the cell lysate of *E. coli* BL21 (DE3) that had been transformed with the pGEMEX-1-HN plasmid (lane 2), the cell lysate of *E. coli* BL21 (DE3) transformed with the pet44a-HN plasmid (lane 3), the purified pGEMEX-1-HN proteins (lane 4), the pet-44a-HN proteins (lane 5) and the cell lysate of *E. coli* BL21 (DE3) transformed with control plasmid of pGEMEX-1 (lane 6) were separated by 15% SDS-PAGE. The target fusion proteins are indicated by arrows. Following transfer, the proteins were probed with 2μg/ml of anti-HN IgG2a and subsequently detected with HRP-IgG, followed by visualization with enhanced chemiluminescence. Lanes 7 – 11 represent the same proteins in the lanes 2 – 6. The specificity and recognition of anti-HN IgG2a were determined by western blot assay using the synthesized peptide of HN (lane 2) and control BSA (lane 1, the panel B). Data shown are representatives of images from three independent experiments.

least in Western blot assay. Further analysis revealed that the anti-HN IgG interacted with the synthesized peptide in a dose-dependent manner and its Ka reached 2.0×10^8 M^{-1} (Figure 2). Therefore, the mAb of anti-HN IgG generated in this study specifically recognized HN antigen and interacted with the antigen with a high affinity.

HN has been found to regulate the pathogenesis of Alzheimer's disease (AD) and the apoptotic process of many types of cells. HN can interact with Bax, insulin-like growth factor binding protein 3 (IGFBP-3), and formyl peptide receptor-like 1 (FPRL1) to regulate cell growth, migration, and apoptosis (Guo et al., 2003, Ikonen et al., 2003, Ying et al., 2004). However, due to the lack of a specific mAb that recognizes HN, the distribution of HN in rodent and human subjects remains unknown. In this study, we immunized BALB/c mice with the purified fusion protein of pet-44a-HN and generated a hybridoma cell clone that stably secreted high levels of anti-HN IgG2a. Analysis of specificity and affinity revealed that this mAb specifically recognized HN and the fusion proteins of pet-44a-HN and pGEMEX-1-HN, but not *E. coli* proteins and control BSA. Furthermore, this mAb interacted with HN with a Ka of 2.0×10^8 M^{-1}. These unique features of this mAb make it suitable for detection of HN specifically and sensitively. Using this mAb, we might be able to determine the pattern of HN expression in rodents and humans. If this mAb can neutralize the interaction of HN with Bax, IGFBP-3, and FPRL1, it may be used as a therapeutic regiment for promoting tumor cell apoptosis.

HN is a small peptide with low immunogenicity, which makes it difficult to induce a strong immune response. In this study, we immunized BALB/c mice with the purified fusion protein of pet-44a-HN and successfully generated hybridoma clones that stably produced HN-specific mAb. This experimental strategy was easily performed and avoided the complex procedure of peptide conjugation with a carrier protein and subsequent purification, which is a procedure commonly used for generating high immunogenicity of peptide antigens for immunization. In

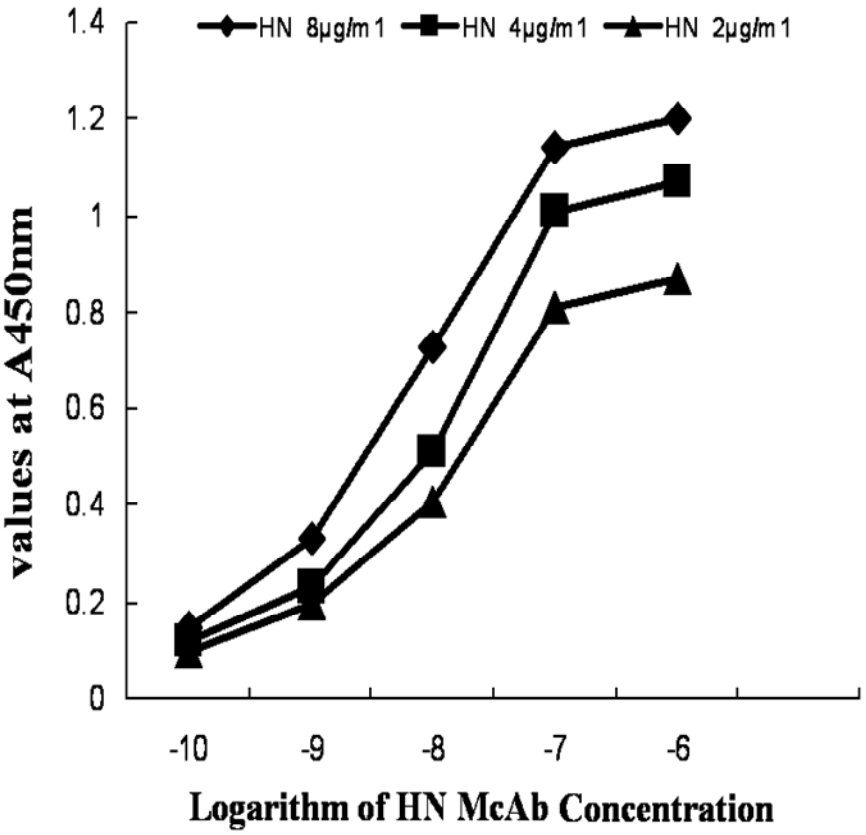

Figure 2. ELISA analysis of anti-HN IgG2a. The recognition of anti-HN IgG2a was determined by indirect ELISA using the indicated concentrations of HN peptide as the coating antigen and anti-HN for antibody detection. The plate wells coated with BSA or coated with HN peptide and probed with isotype control IgG2a were used as negative controls. The optical density values of control wells were around 0.05 – 0.1. Data shown are the mean values from three separate experiments and the intra-group variation was less than 10% of the mean value.

addition, we used the synthesized peptide as the coated antigen for ELISA, which led to specific detection of anti-HN hybridoma clones. Therefore, application of the purified fusion protein for immunization and the synthesized peptide as the antigen for *in vitro* screening of hybridoma clones is feasible and effective for generation and characterization of anti-peptide antigen-specific mAbs. The mAbs we prepared in the current study have been successfully used in ELISA and western blot analysis. However, whether it could be used for immunohistochemical staining and flow cytometry analysis and have neutralization activity remains to be further determined.

In summary, we successfully generated hybridoma clones that stably produced HN-specific mAbs. The mAb 5A8H3 was an IgG2a that interacted with HN specifically and sensitively. To the best of our knowledge, this was the first mAb against HN reported in the world. Conceivably, this mAb can be a useful tool for further investigation of the expression and function of HN in rodents and humans.

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Full Length Research Paper

Genetic structure and diversity within and among six populations of *Capparis decidua* (forssk.) edgew. from Saudi Arabia

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Capparis decidua **is a rangeland plant species growing in isolated populations in Saudi Arabia. Genetic diversity within and among six populations (Madina, Farasan island, Hawayer Assos, Khor Assos, Raudhat Khuraim, and Taif) of *C. decidua* was studied using RAPD technique. Of the 25 random primers were used, eighteen (18) primers generated discernible and reproducible bands. A total of 152 reproducible RAPD bands across the 36 individuals were amplified. Out of those, 117 (76.2%) RAPD bands were polymorphic. The number of polymorphic bands per primer ranged between 3 and 11 with an average of 6.5 bands per primer. Populations differed in the level of genetic diversity as shown from the percentage of polymorphic bands. Farasan population had the highest level of genetic diversity (24.3%) and two populations Khor Assos (5.9%) and Taif (4.6%) had the lowest genetic diversity. Analysis of molecular variance (AMOVA) showed highly significant differences among populations. Among the population variance accounted, there is a higher percentage of the total variance (average 77.67%, SD±8.21) than within populations (average 22.33%, SD±8.21). There is no significant correlation between geographical distance and genetic distance was found. However, there was a significant positive correlation between molecular genetic variation and actual population size. The implication of the results of this study in devising strategy for conservation of *C. decidua* is discussed.**

Key words: *Capparis decidua*, Tandhab, Assos, Population size, RAPD markers, Genetic diversity.

INTRODUCTION

An increasing number of plant species have been restricted to small and isolated populations due to the habitat destruction and fragmentation. These populations face an increased risk of extinction because of environmental, demographic and genetic stochastic threats, even in intact habitats (Fisher and Matthies, 1998). Random fluctuation of environmental conditions that affect plant survival and reproduction are considered to be the most important stochastic factors (Boyce, 1992; Menges, 1992), whereas, demographic stochastity in infinite populations is considered to be of minor importance (Menges, 1991).

Knowing the degree of genetic variation is of fundamental importance for species' conservation (Barrett and Kohn, 1991; Ellstrand and Elam, 1993; Gilpin and Soule, 1986; Hamrick and Godt, 1996a; Karron, 1997; Lande, 1999). Positive correlations between fitness-related characters and heterozygosity have been found in a number of plant

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Abbreviations: RAPD, Random amplified DNA; **CTAB,** cetyltrimethyl ammonium bromide; **AMOVA,** analysis of molecular variance; **UPGMA,** unweighted Pair group method with arithmetic mean; **SD,** standard deviation.

species (Linhart and Mitton, 1985; Oostermeijer et al.,1995). Small populations always have low genetic diversity; consequently, their capacity to adapt to environmental change may be diminished. In addition, their ability to survive over the long term may be compromised (Lande, 1999).

Inbreeding and genetic drift affect population fitness through the increased expression of recessive deleterious alleles as homozygosity increases in small populations (Ellstrand and Elam, 1993; Karron, 1997; Lande, 1999). Genetic drift is expected to randomly reduce variation within small populations, causing loss of low frequency alleles, which can be associated with population fitness (Barrett and Kohn, 1991). Moreover, the random loss of self-incompatibility alleles may reduce the reproductive capacity of individuals (DeMauro, 1993; Karron, 1997; Young et al., 1999) and may lead to population extinction (Barrett and Kohn, 1991; Holsinger et al., 1999). Gilpin and Soule (1986) stated that, in small populations these genetic factors combined with demographic stochasticity, may result in ‘‘extinction vortices,’’ feedback process that result in reducing the number of individuals until populations become extinct. Although, the above discussion is true, not all small populations are genetically going through ‘‘extinction vortices’’ (Ellstrand and Elam, 1993; Frankham 1997; Gitzendanner and Soltis, 2000; Godt and Hamrick, 1998). There are other factors such as species’ life-history, biogeography, and *gene* flow into the population that could also play critical roles in determining the current genetic composition of populations (Hamrick and Godt, 1996a,b; Holsinger et al., 1999). Therefore, understanding genetic factors that contribute to extinction risks for particular species is critically important for their conservation (Godt and Hamrick, 1998; Hamrick and Godt, 1996a).

Capparis deciduas (Forssk.) Edgew., family *Capparidaceae* (vernacular name; Tandhab, Assos) is a bushy shrub occurring in dense tufts. It reaches a height of 4-5 m or more. The species is common in dry tropical areas of Africa and Asia. It is an important plant because of its excellent adaptation to arid conditions. *C. decidua* was found to be one of the best species for shelter belts to check the movement of sand in the Thar desert of India (Pandey and Rokad, 1992). As a drought resistant, it has relatively good nutritive value (Assaeed et al., 1995) and withstands neglect. In Saudi Arabia, *C. decidua* is present in isolated populations over much of the country and some are limited in size (Miller and Cope, 1996; Collenette, 1985). The plant is also heavily browsed by camels and goats. Being under biotic and abiotic stresses, it is feared that genetic diversity of *C. decidua* populations in Saudi Arabia may decrease. The objectives of this study were to: (1) analyze the genetic diversity within and among six populations occurring over a wide area in Saudi Arabia with RAPD markers, (2) relate the population size to genetic diversity and (3) determine if the geographic distance is related to genetic similarity among populations.

MATERIALS AND METHODS

Population sampling

Sampling included materials from six populations (Madina, Farasan Island, Hawayer Assos, Khor Assos, Raudhat Khuraim, and Taif). Details of the locations of these populations are given in Table 1 and Figure 1. Six randomly chosen individuals from each of the six populations were sampled in spring 2005. Fresh twigs of current year growth of *C. decidua* were collected, placed into plastic sealable bags and transported to the laboratory in an ice box. Samples were washed several times with distilled water before being subjected to lyopholization. Lyophilized material was kept at -20°C until isolation of DNA.

Genomic DNA extraction

Lyopholized samples were ground to a fine powder. DNA extraction was extracted from 20 - 50 mg of powdered material using a common CTAB procedure (Doyle and Doyle, 1990) modified by adding 5% polyvinylpyrrolidone (w/v) and 2% 2-mercaptoethanol (v/v) to the extraction buffer. The DNA concentration was determined either by electrophoresis on 1% agarose gel stained with ethidium bromide with comparisons made to standard DNA ladders or by spectrophotometer readings at 260/280 nm. The DNA samples were diluted to 25 ng/µl and kept at -20°C until use.

RAPD assay

Twenty five different RAPD primers were initially screened in this study. PCR amplification was carried out in a 25 µl reaction mixture with the following components: 1x supplied PCR buffer (10 mM Tris–HCl pH 9, 50 mM KCl, 2 mM $MgCl_2$ and 0.01% Triton X-100), 1 U FastStart *Taq* DNA polymerase (Roche), 200 mM of each dNTP, 30 nM of primer and 25 ng of DNA template. Optimal amplification conditions for RAPDs were one cycle of 15 min at 94°C, followed by 45 cycles of 30 s at 94°C, 1 min annealing at 36°C and 2 min at 72°C followed by a final step of 10 min at 72°C. Samples were cooled at 4°C until recovery. RAPD fragments were separated in 2.0% agarose gel, stained in ethidium bromide (0.5 µg/ml) and visualized by UV trans-illumination. For band sizing DNA molecular weight standard *Gene* Ruler (100 bp ladder, Fermentas Life Sciences, USA) was used.

Data analysis

To examine the genetic relationship within the population, an UPGMA dendogram based on Nei’s coefficient of genetic similarity (Lamboy, 1994) was constructed using a SIMQUAL, SAHN and TREEPLOT routines as implemented by NTSYS-pc, Version 2.02c (Rohlf, 1997). The PCR polymorphisms data generated from the thirty six individual plants were scored into binary matrices indicating absence (0) or presence (1) of particular RAPD fragment. Analysis of molecular variance (AMOVA) was carried out using Arlequin software 3.0 (Schneider et al., 2000) to statistically test the existence of differences among six *C. decidua* populations. All possible 15 pair wise population group comparisons were defined for AMOVA test. The significance level for AMOVA calculations was 0.01. Correlation co-efficients for the linear association between

Table 1. Brief description of *C. decidua* populations.

Population	Population size	Percentage of polymorphic bands	Longitude, latitude, and altitude	Associated species
Madina	45	19.7	24º 08' N 39º 34' E 761 m	*Acacia asak*, *A. tortilis*, *Ochradenus baccatus*, *Lycium shawii*, *Aerva tomentosa*, *Leptadenia pyrotechnica* and *Belpharis ciliaris.*
Farasan	950	24.3	16º 39' N 40º 29' E 7 m	*A. ehrenbergiana*, *Commiphora opobalsamum*, and *Salvadora persica.*
Hawayer Assos	35	15.1	25º 11' N 48º 37' E 357 m	*A. gerrardii, Calotropis procera, L. shawii* and *O. baccatus*
Khor Assos	53	5.9	26º 59' N 45º 33' E 524 m	*A. gerrardii* and *L. shawii* *ochradenus baccatus*
Raudhat Khuraim	183	7.9	25º 26' N 47º 14' E 556 m	*Ziziphus nummularia*, *A. tortilis*, *A. ehrenbergiana*, *A. gerrardii*, *L. shawii*, *C. procera*, *Pulicaria crispa*, *Achillea fragrantissima*, *Zilla spinosa*, *Lasiurus scindicus* and *Pennisetum divisum*
Taif	85	4.6	21º 18' N 40º 29' E 1577 m	*A. asak, A. tortilis, A. flava, L. persicum, Tamarix articulata, O. baccatus, P. crispa, Aerva tomentosa, Coccinia grandis, Cenchrus ciliaris* and *Panicum turgidum*

Figure 1. Map showing the locations of six analyzed populations of *C. decidua* in Saudi Arabia: Khor Assos, Raudhat Khuraim, Hawayer Assos, Medina, Taif and Farasan.

Table 2. Primer sequences, total number of amplified fragments for each primer, number of polymorphic bands and percentage of polymorphism averaged over the six populations.

Primer name	Primer sequence	Number of bands	Polymorphic bands	Percentage of polymorphism
OPA-01	CAGGCCCTTC	10	7	70.0
OPA-02	TGCCGAGCTG	11	8	72.7
OPA-03	AGTCAGCCAC	10	9	90.0
OPA-04	AATCGGGCTG	13	11	84.6
OPA-05	AGGGGTCTTG	8	7	87.5
OPA-06	GGTCCCTGAC	5	3	60.0
OPA-08	GTGACGTAGG	8	6	75.0
OPA-09	GGGTAACGCC	10	7	70.0
OPA-12	TCGGCGATAG	11	8	73.0
OPA-13	CAGCACCCAC	8	7	87.5
OPA-14	TCTGTGCTGG	7	5	71.4
OPA-16	AGCCAGCGAA	6	5	83.0
OPA-17	GACCGCTTGT	6	3	50.0
OPA-18	AGGTGACCGT	5	4	80.0
OPB-01	GTTTCGCTCC	7	5	71.4
OPB-02	TGATCCCTGG	7	6	85.7
OPB-03	CATCCCCCTG	11	9	81.8
OPB-04	GGACTGGAGT	9	7	77.8
Total		152	117	76.2

percentage of polymorphic bands and population size and between Nei's genetic similarity and geographical distance were calculated according to Steel and Torrie (1980).

RESULTS

RAPD and statistical analysis

A total of 25 random 10-mer primers (Operon Co., USA) were screened against six individuals per population. Of these, 18 primers that could generate discernible and reproducible bands were selected for further amplification (Table 2). A total of 152 reproducible RAPD bands were generated with the 18 primers across the 36 individuals of the six populations of *C. decidua*. Out of these RAPD bands, 117 (76.2%) were polymorphic (Table 2). The bands ranged in size from 220 - 2000 bp. The number of polymorphic bands per primer ranged between 3-11 with an average of 6.5 bands per primers (Table 2).

Primers varied in their ability to detect variation both within and between populations. Some primers showed polymorphism in some population, and were monomorphic in other populations. These differences suggested that a sufficient number of primers are essential for a reliable evaluation of the genetic diversity.

Populations differed in the level of genetic diversity as shown from the percentage of polymorphic bands in each population. For example, Farasan had the highest level of genetic diversity (24.3%) followed by Madina (19.7%). Hawayer Assos (15.1%) and Raudhat Khuraim (12.9%) had a moderate genetic diversity. However, both Khor Assos (5.9%) and Taif population (4.6%) had the lowest genetic diversity.

The genetic relationship of the 36 individuals in the six populations was illustrated using the UPGMA dendrograms based on Nei's genetic similarity coefficient (Figure 2). The six populations are clustered into two major groups, each of them carrying three subgroups. Madina, Farasan, and Hawayer Assos populations formed a very closely related group. The second group includes Khor Assos, Raudhat Khuraim, and Taif. The last two populations are very closely related and the most related of the six populations. According to the dendrogram, Madina and Taif populations are the most different of all the six populations.

To analyze genetic structure within and among populations, AMOVA analysis (Table 3) was performed for 6 different populations (15 pair-wise calculations). In all cases, AMOVA test resulted in highly significant ($p < 0.01$) differences between populations. The variance among populations accounted for a higher percentage of the total variance (average 77.67%, SD±8.21) than the variance within populations (average 22.33%, SD±8.21).

DISCUSSION

This study provides the first detailed analysis of genetic variability of *C. decidua* in Saudi Arabia. Previously, genetic variability was conducted in just one population using RAPD markers (Abdel-Mawgood et al., 2005; 2006). RAPD markers, along with appropriate statistical procedures,

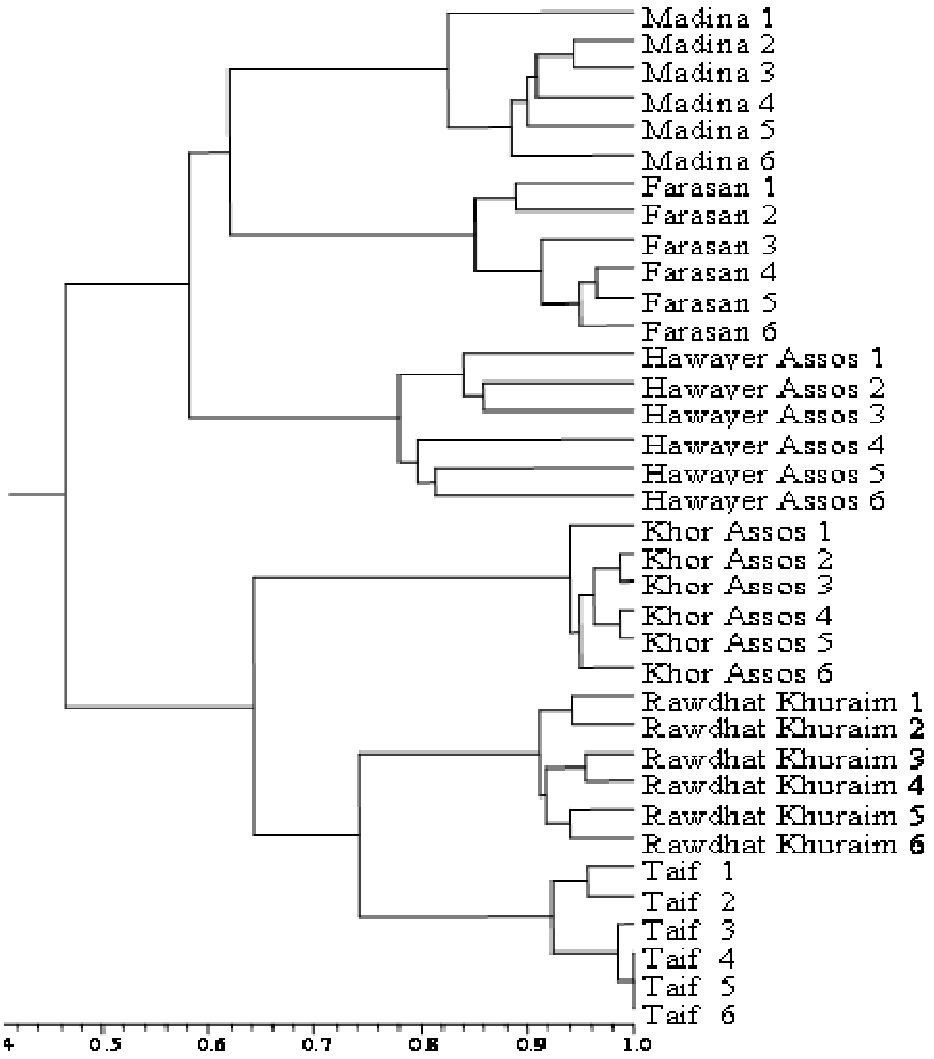

Figure 2. UPGMA dendrogram of 36 *C. decidua* plants based on Nei's genetic similarity.

Table 3. Matrix of mean Nei's unbiased genetic similarity [a] and in parentheses-pairwise geographical distance [b] (below diagonal) and percentage of total variation (between [c] and within [d] populations) values from AMOVA analysis for pair wise comparisons among population (above diagonal).

	Madina	Farasan	Hawayer Assos	Khor Assos	Raudhat Khuraim	Taif
Madina		58.81[c] (41.19)[d]	68.85 (31.15)	82.22 (17.78)	82.61 (17.39)	85.32 (14.68)
Farasan	0.651[a] (867.7) [b]		63.79 (36.21)	76.77 (23.23)	75.55 (24.45)	77.29 (22.71)
Hawayer Assos	0.621 (918.0)	0.587 (1150.5)		83.44 (16.56)	81.22 (18.78)	84.24 (15.76)
Khor Assos	0.518 (671.8)	0.462 (1185.4)	0.487 (365.8)		81.90 (18.10)	87.49 (12.51)
Raudhat Khuraim	0.423 (780.4)	0.401 (1099.9)	0.458 (143.1)	0.663 (242.6)		75.56 (24.44)
Taif	0.427 (325.5)	0.449 (543.8)	0.449 (931.2)	0.631 (808.9)	0.736 (822.7)	

are suitable for genetic variation analysis at both intra- and inter-population levels and for devising strategies for conservation (Sun and Wong, 2001; Pvingila et al., 2005; Li et al., 2008; Huang et al., 2008).

Genetic diversity

The of eighteen RAPD primers used in this study were highly informative and produced 152 bands with an

average of 8.4 bands per primer. Of these bands, 117 were polymorphic across all populations (67.2% polymorphic bands) reflecting divergence between populations. The remaining bands were common in all populations. The level of genetic diversity for *C. decidua* is higher than expected based on geographical distribution and when compared with other plant species with similar life histories. The percentage of polymorphic bands in endangered plant species is reported to be 69% in *Changium smyrnioides* (Fu et al., 2003), 24.5% in *Lactoris fernandezziana* (Lactoridaceae), (Brauner et al., 1992), 22.5% in *Paeonia suffruticosa*, 27.6% in *P. rockii* (Pei et al., 1995), and 33% in *Dacydium pierrei* (Su et al., 1999). One possible explanation for high variability is the out crossing or hybridization between individual plants within a population.

It may be an important component of recent evolution and may also be a factor contributing to the high level of genetic diversity. Moreover, the high number of polymorphic products generated by some primers- as shown in our data- might be attributed to the fact that in RAPD, there are various reasons that can result in polymorphism, which are well documented (Scott and Crandall-Stotler, 2002; Juchum et al., 2007; Ge et al., 1999).

Genetic structure

Genetic structure of plant population reflects the interactions of various factors such as long term evolutionary history of species, genetic drift, mating system, *gene* flow and selection (Schaal et al., 1998). Farasan population was the most genetically diverse population with 24.3% polymorphic bands. The least genetically diverse populations were Khor Assos and Taif with percentages of polymorphic bands of 5.9 and 4.6, respectively (Table 1).

Geographical distances between each pair of the studied populations vary from 143 - 1185 km (Figure 1). No significant correlation (r=0.15) was detected between the genetic and geographical distances among the six populations. However, some populations show certain correlation between genetic distance and geographical distance. For example, according to the dendrogram (Figure 2), Khor Assos and Raudhat Khuraim were clustered in one group and they are geographically very close, with 246 km apart. It is possible that these two populations have had the same origin and still could maintain some level of *gene* flow between them. In contrast, Raudhat Khuraim and Taif were grouped together despite the long geographical distance of 822 km between them. The same is true for Khor Assos and Taif populations. The lack of such correlation could be partially explained by possible adaptive eco-geographical differentiation associated with habitat fragmentation or by severe bottlenecks in some populations in the past (Owuor et al., 1997). These could possibly result in forming a new allelic composition of the populations irrespective of their geographical location (Gaudeul et al., 2000).

Correlation between population size and genetic diversity

There was a significant positive correlation (r = 0.64) between molecular genetic variation and actual population size in the six populations. The low level of genetic variability in smaller size populations and the observed correlation is most likely due to the loss of variation in small population through genetic drift. Similar results were obtained for small-size isolated populations in several other studies. For example, population size was significantly correlated with the proportion of polymorphic loci in the out breeding perennials *Saliva pratensis* and *Scabiosa columbaria* (Van Treuren et al., 1991). Another study covering 25 populations of a rare perennial plant, *Gentiana pneumonanthe*, showed similar results (Raijmann et al., 1994. In contrast, Dixon and May (1990) found no consistent relationship between heterozygosity and population size in *Aconitum noveboracense*.

Implication for conservation

As stated by Banares et al. (1993), "the main objectives of a recovery plan are the preservation of genetic diversity, and to insure the continuous survival of populations especially by the perpetuation of species in their natural habitat without any specific human aid to maintain evolutionary potential". Information on current level of genetic diversity of a population is essential for designing appropriate strategies for conservation (Falk and Holsinger, 1991). The results of the current study showed that there is a positive correlation between population size and percentage of polymorphic loci. Moreover, most of these populations are small (with the exception of Farasan population) ranging in size from 35 - 183 trees (Table 1). In addition, these populations are isolated and fragmented.

Ellstrand and Elam (1993) indicated that in isolated populations, genetic drift may reduce genetic variation, increase levels of inbreeding and consequently, reduce the potential to adapt to environmental changes. Reduced level of genetic diversity can affect plant fitness and population persistence in several ways. In the long term, populations with reduced level of genetic diversity have low potential for adaptation to changes in environmental conditions. While in the short term, low level of genetic diversity may affect fitness through increased level of inbreeding and inbreeding depression. This may suggest that these populations may be of particular conservation concern as it is unlikely for them to recover from any stochastic extinction events.

The results of this study could be meaningful for devising strategy for conservation of *C. decidua*. For *ex-situ* and *en-situ* program, sampling should be representative of all populations giving the high level of within population diversity. Moreover, preservation of genetic

variation should be through conservation of a large number of individuals especially for small size populations such as Madina, Hawayer Assos, and Khor Assos. In conclusion, it seems, thus, advisable to give to *C. decidua* a conservation priority on ecological grounds.

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Full Length Research Paper

Optimization of fermentation medium for nisin production from *Lactococcus lactis* subsp. *lactis* using response surface methodology (RSM) combined with artificial neural network-genetic algorithm (ANN-GA)

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Nisin is a bacteriocin approved in more than 50 countries as a safe natural food preservative. Response surface methodology (RSM) combined with artificial neural network-genetic algorithm (ANN-GA) was employed to optimize the fermentation medium for nisin production. Plackett-Burman design (PBD) was used for identifying the significant components in the fermentation medium. After that, the path of steepest ascent method (PSA) was employed to approach their optimal concentrations. Sequentially, Box-Behnken design experiments were implemented for further optimization. RSM combined with ANN-GA were used for analysis of data. Specially, a RSM model was used for determining the individual effect and mutual interaction effect of tested variables on nisin titer (NT), an ANN model was used for NT prediction, and GA was employed to search for the optimum solutions based on the ANN model. As the optimal medium obtained by ANN-GA was located at the verge of the test region, a further Box-Behnken design based on the RSM statistical analysis results was implemented. ANN-GA was implemented using the further Box-Behnken design data to locate the optimum solution which was as follow (g/l): Glucose (GLU) 15.92, peptone (PEP) 30.57, yeast extraction powder (YEP) 39.07, NaCl 5.25, KH_2PO_4 10.00, and $MgSO_4 \cdot 7H_2O$ 0.20, with expected NT of 22216 IU/ml. The validation experiments with the optimum solution were implemented in triplicate and the average NT was 21423 IU/ml, which was 2.13 times higher than that without ANN-GA methods and 8.34 times higher than that without optimization.

Key words: Response surface methodology, artificial neural network, genetic algorithm, nisin titer.

INTRODUCTION

Nisin is a bacteriocin produced by molecular strains of *Lactococcus lactis* subsp. *lactis*. It is composed of 34 amino acids and approved in more than 50 countries as a natural food preservative for various products such as processed cheese, beverages, canned foods, etc. Nisin is reported to act primarily upon the cytoplasmic membrane, and is effective against a broad range of Gram-positive bacteria and their spores. Besides, it can also inhibit the growth of a wide variety of Gram-negative bacteria with ethylenediaminetetraacetic acid (EDTA) (Cheigh and Pyun, 2005; Delves-Broughton et al., 1996; Economou et al., 2009; Soriano et al., 2004). The nisin precursor is synthesized in the early active growth phase, the nisin production rate is maximal towards the end of the exponential growth phase, and cells completely stop nisin biosynthesis when entering the stationary growth phase. Therefore, nisin production is greatly influenced by the growth of bacteria and the fermentation media (Liu et al.,

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2005; De Vuyst and Vandamme, 1993; Mirdamadi et al., 2008; Wardani et al., 2006). Optimal nisin production in batch culture usually requires a complex fermen-ation media. Several literatures presented studies on the optimization of fermentation media for nisin production using statistical methods, such as response surface methodology (RSM), multi-linear regression and logistic model. However, these methods are not suitable for such complex system, and it is difficult to develop satisfying models with high predictive accuracy using these methods (Lv et al., 2004; Lv et al., 2005; Penna and Moraes, 2002; Vazquez and Murado, 2008).

RSM is a collection of statistical techniques for desig-ning experiments, developing models, evaluating the effects of factors, and searching for optimum conditions. Plackett-Burman design (PBD) is a 2-level experimental design that requires fewer runs than a comparable fractional design. It can be used to identify the significant factors among many candidate factors. The path of steepest ascent (PSA) is a procedure for moving sequen-tially along the path of the steepest ascent, that is, in the direction of the maximum increase in the response. Box-Behnken design and multi-quadric regression could find out the relationship between the variables and response values based on statistical analysis, and the optimum of each variable would be obtained as well. RSM has been extensively used in the optimization of medium composition, conditions of enzymatic hydrolysis, fermentation and food manufacturing processes (Choudhari and Singhal, 2008; Ghosalkar et al., 2008; Mannan et al., 2007; Wang and Liu, 2008; Wu et al., 2007).

Artificial neural network (ANN) is a non-linear com-putational model based on biological neural networks. It simulates the human brain learning process by mathema-tically modeling the network structure of interconnected node cells. ANN has been utilized with high success for system design, modeling, optimization and control mainly due to their capacity to learn, filter noisy signals and generalize information through a training procedure. Several literatures have demonstrated that the predictive accuracy of ANN models were superior to RSM model using the same experimental design. The advantages of ANN compared to RSM are: (i) ANN does not require a prior specification of suitable fitting function and (ii) ANN has universal approximation capability, that is, it can approximate almost all kinds of non-linear functions including quadratic functions, whereas RSM is useful only for quadratic approximations. However, ANN is known as a black box modeling approach. Therefore, the effect of factors on response values and the interaction effect among the factors cannot be studied by ANN model. Genetic algorithm (GA) which is an artificial intelligence based stochastic non-linear optimization formalism, is used to optimize the input space of the ANN model. This hybrid methodology will be referred to as ANN-GA hereafter. The GA mimics the principles of biological evolution, namely “survival-of-the-fittest” and “random exchange of data during propagation” followed by biologically evolving species. GA has been considered an ideal technique to solve diverse optimization problems in biochemical engineering. In the present work, RSM and ANN-GA were applied to optimize the fermentation media for nisin production simultaneously. This method can much more efficaciously evaluate both the effect of the individual factor and the interaction effect among the factors, and increase the nisin titer (NT) as well (Chen et al., 2004; Desai et al., 2006; Moreira et al., 2007; Nagy, 2007; Singh et al., 2008; Tramer and Fowler, 1964).

MATERIALS AND METHODS

Microorganism and seed culture preparation

A mutant strain *L. lactis* subsp. *lactis* CGMCC NO. 3050 obtained by treating *L. lactis* subsp. *lactis* ATCC 11454 with diethyl sulfate (DES) was used for the optimization studies. *L. lactis* subsp. *lactis* ATCC 11454 was purchased from the American Type Culture Collection. *L. lactis* subsp. *lactis* CGMCC NO. 3050 was collected in China General Microbiological Culture Collection Center. It was maintained on CM slants medium composed of (g/l): Glucose (GLU) 10, peptone (PEP)10, yeast extraction powder (YEP)10, KH_2PO_4 10, NaCl 2, $MgSO_4 \cdot 7H_2O$ 0.2 and agar 15. The medium was adjusted to pH 6.8 using 5 M NaOH before autoclaving at 121°C for 30 min. GLU was separately autoclaved at 105°C for 10 min. The slants were inoculated to 3 ml sterile CM liquid medium in cuvette (15 ml) and incubated at 37°C in a rotary shaker at 150 rpm for 24 h. Then, the culture was inoculated to Erlenmeyer flask (250 ml) containing 100 ml sterile CM liquid medium, which was incubated at 37°C in a rotary shaker at 150 rpm for 18 h, and the seed culture was prepared.

Production media and cultivation condition

The initial medium for nisin production was CM medium. The concentrations of the components in CM medium were varied depending on the experimental design used for nisin production fermentation. 3% (v/v) inoculum was added aseptically to Erlenmeyer flasks (250 ml) containing 100 ml of designed medium. The fermentation medium was incubated at 37°C in rotary flasks at 150 rpm for 24 h.

Analytical methods

NT was measured by a modified method of Tramer and Fowler (1964). The fermentation broth was adjusted to pH 2 using 10 M HCl, and then centrifuged at 5000 rpmin for 10 min. The supernatant was appropriately diluted with 0.02 M HCl and nisin assay were performed by an agar well diffusion assay. A standard curve (200 - 3000 IU /ml) was plotted using a stock solution of 4000 IU/ml nisin (Sigma, USA). Each assay was performed in triplicate and the average result was presented.

PBD

PBD experiments had 9 factors and 2 levels, employed to select the Significant factors based on statistical analysis. Among the 9 factors, six were the components in the CM medium. The high (+1) and low (-1) level of the six components were GLU (X_1) 12.5 and 10.0 g/l; PEP (X_2) 12.5 and 10.0 g/l; YEP(X_3) 12.5 and 10.0 g/l; KH_2PO_4 (X_4) 12.5 and 10.0 g/l; NaCl (X_5) 2.5 and 2.0 g/l; $MgSO_4 \cdot$

$7H_2O$ (X_6) 0.25 and 0.20 g/l. The other three factors were dummy variables (X_7, X_8, X_9), used for estimating the experimental error and checking the adequacy of the first-order model. Twelve experimental runs were carried out in the present work. Then, a first-order model in coded variables was obtained using SAS (version 8.02) for the regression analysis of the experimental data. At the end of this stage, GLU, PEP, YEP, and NaCl were screened out as significant factors that impact the NT.

PSA method

PSA experiments were applied to approach the optimal regions of the significant factors while the non-significant were set at the lowest level. PSA started from the center of PBD and moved along the path in which the concentration of GLU, PEP, YEP and NaCl increased according to their coefficients in the above mentioned first-order model.

Box-Behnken design

Box-Behnken design was used for further optimization of fermentation media. The low (-1), middle (0) and high level (1) of GLU (U_1), PEP (U_2), YEP (U_3) and NaCl (U_4) in Box-Behnken design were 13.2, 23.2 and 33.2 g/l ; 12.6, 22.6 and 32.6 g/l; 7.2, 17.2 and 27.2 g/l; 4.4, 6.4 and 8.4 g/l, respectively.

The integration of RSM and ANN for modeling

In RSM modeling, the second-order polynomial coefficients were calculated using SAS 8.02 to estimate the responses of the dependent variables. Multilayer perceptron (MLP) is a commonly used feed-forward ANN model that maps sets of input data onto a set of appropriate output data. In this work, the neural network architecture had three layers, namely the input, hidden and output layer. The concentrations of the significant components in CM medium (coded data) were used as the input nodes, and the NTs were used as the output nodes.

The number of hidden nodes greatly affects the capabilities of the ANN model. Generally, when the number of hidden nodes increases, the ANN model fits the training set better. However, too many hidden nodes will lead to ANN model over-fitting. In order to avoid the over-fitting of ANN model, a criterion named the degree of approximation (D_a) (Guo et al., 2006) was employed to select the suitable number of hidden nodes, as defined in Equation 1

$$D_a = \frac{c}{\frac{n_c}{n} \times MSE_c + \frac{n_t}{n} \times MSE_t + \left| MSE_c - MSE_t \right|} \quad (1)$$

Where, MSE_c and MSE_t are the mean-square-errors (MSE) of calibration set and validation set, respectively; n_c and n_v, number of calibration set and validation set; n, sum of the number of calibration set and validation set; c, a constant number (in the present work c was 1000) by which D_a was adjusted to get a good chart.

According to the above equation, it was obvious that the larger D_a obtained, the more the ANN models approached the experimental data. Since D_a achieved the maximum value when there were 15 hidden nodes, the number of hidden nodes was set to 15.

Training of ANN was minimized by adjusting the network weights appropriately using Levenberg-Marquardt algorithm. The ANN model was trained with Levenberg-Marquardt algorithm for 1000 interations. The MSE function, a commonly employed error function, was used in this work and defines as in Equation 2:

$$MSE = \frac{\sum_{i}^{n} \left(y_{BPANNi} - y_{ACTUi} \right)^2}{n} \quad (2)$$

Where, n referred to the number of patterns used in the training; i denotes the index of the input pattern (vector); y_{ACTUi} and y_{BPANNi} were the desired and predicted outputs of the nth output node, respectively.

In the present work, the ANN model with a fixed topology structure was developed by neural network fitting tool (nftool) in Matlab 7.6.0. It was trained thirty times with the initial weight given randomly and the best ANN model were selected according to their D_a.

Optimization by ANN-GA

After the optimum ANN model was developed, GA was employed to search for the optimum solution in the input space of the ANN model, which was implemented in Matlab 7.6.0. The values of GA-specific parameters used in the optimization simulations were: Population type as double vector, population size as 20, the initial population as given randomly, selection function as stochastic uniform, elite count as 2, crossover fraction as 0.8, crossover function as scattered, migration fraction as 0.2, penalty factor as 100 and number of generation over which GA evolved as 100. The fitness function was defined as follows:

$$Fitness = \frac{1000}{NT_{ANN \ \mathrm{mod} \ el}} \quad (3)$$

Where, $NT_{ANN\ model}$ is the NT calculated by ANN model.

Besides, the GA-based optimization simulations were repeated by using each time a different randomly initialized population of the candidate solutions. Dissimilar initial populations ensure that each time, the GA began its search for the optimal solution from a different search sub-space which helped in locating the lowest local or the global minimum on the fitness function surface.

RESULTS

PBD

Base on the initial medium (CM medium), a PBD experiment was developed. The initial NT was 2568.71 IU/ml obtained by the initial medium. The experimental data of PBD are presented in Table 1 and a linear model was employed to fit these data obtained using SAS 8.02. The first-order model is shown in Equation 4 and the statistical analysis results are shown in Table 1. The coefficient of determination (R^2) was 0.9847, indicating that 98.47% of the variability in the response could be explained by the model. The statistical significance of this model was evaluated by the F-test analysis of variance which reveals that this regression was statistically significant ($P < 0.1$) at 90% confidence level. The F-test was used for identifying the effect of candidate factors on NT. The concentrations of GLU (X_1), PEP (X_2), YEP (X_3) and NaCl (X_5) were selected as significant factors.

Table 1. The matrix of the PBD and the experimental results.

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	NT (IU/ml)
1	1	-1	1	-1	-1	-1	1	1	1	1682
2	1	1	-1	1	-1	-1	-1	1	1	1937
3	-1	1	1	-1	1	-1	-1	-1	1	2079
4	1	-1	1	1	-1	1	-1	-1	-1	1937
5	1	1	-1	1	1	-1	1	-1	-1	2570
6	1	1	1	-1	1	1	-1	1	-1	2231
7	-1	1	1	1	-1	1	1	-1	1	1805
8	-1	-1	1	1	1	-1	1	1	-1	1460
9	-1	-1	-1	1	1	1	-1	1	1	890
10	1	-1	-1	-1	1	1	1	-1	1	1682
11	-1	1	-1	-1	-1	1	1	1	-1	1360
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	829
F values	49.303	46.278	13.984	2.043	6.992	1.602	1.618	6.788	0.371	--
p values	0.020	0.021	0.065	0.289	0.118	0.333	0.331	0.121	0.605	--

Table 2. Experimental design and results of PSA.

Run	GLU (g/l)	PEP (g/l)	YEP (g/l)	NaCl (g/l)	Nisin titer (IU/ml)
1	11.2	11.2	11.2	2.2	3947
2	13.2	13.1	12.2	2.9	4236
3	15.2	15.0	13.2	3.6	4546
4	17.2	16.9	14.2	4.3	6030
5	19.2	18.8	15.2	5.0	6946
6	21.2	20.7	16.2	5.7	8000
7	23.2	22.6	17.2	6.4	9214
8	25.2	24.5	18.2	7.1	8586

$$Y=1705.636+301.202X_1+291.818X_2+160.414X_3+61.307X_4+ 113.431X_5-54.296X_6+54.572X_7-111.764X_8-26.118X_9 \quad (4)$$

The PSA method

PSA started from the center of PBD and moved along the path in which the concentration of GLU, PEP, YEP and NaCl increased according to their coefficients. The design and results of PSA experiments are shown in Table 2. As can be seen, the maximum NT was 9214 IU/ml, when the media were GLU 23.2 g/l, PEP 22.6 g/l, YEP 17.2 g/l, and NaCl 6.4 g/l, respectively. It was suggested that this point is near the region of maximum response.

The integration of RSM and ANN for modeling

The Box-Behnken design matrix and the corresponding experimental data are shown in Table 3. A multi-quadratic model in coded variables and a two-layer feed forward ANN were applied to fit the Box-Behnken design simultaneously. The multi-quadratic model was as follows:

$$Y=9214.551-511.186U_1+1806.975U_2+1790.820U_3-505. 1235.416U_2^2\ 445.821U_2U_3-1169.692U_2U_4-1456.222U_3^2 .839U_4-1603.912U_1^2+677.520U_1U_2-1491.210U_1U_3+418.087U_1U_4- ^2+354.405U_3U_4-2620.689U_4^2 \quad (5)$$

The statistical analysis results of RSM model are shown in Table 4. According to Table 4, the smaller the magnitude of the P-value, the more significant the corresponding coefficient. The P-values that were less than 0.05 indicating corresponding model terms were significant. The coefficient estimates and the corres-ponding P-values suggested that among the test factors, U_2 (PEP), U_3 (YEP), U_1U_3 (interaction effect between GLU and YEP),

Table 3. The matrix of Box-Behnken design and experimental results.

Run	U_1	U_2	U_3	U_4	Experimental NT (IU/ml)	$NT_{RSM\ model}$[1] (IU/ml)	$NT_{ANN\ model}$ (IU/ml)
1	-1	-1	0	0	6946	5757	6937
2	-1	1	0	0	8000	8016	8747
3	1	-1	0	0	4236	3380	4252
4	1	1	0	0	8000	8349	7983
5	0	0	-1	-1	4878	4207	4868
6	0	0	-1	1	2407	2487	2419
7	0	0	1	-1	8000	7080	8013
8	0	0	1	1	6946	6777	6564
9	-1	0	0	-1	5619	6425	5621
10	-1	0	0	1	4878	4577	4890
11	1	0	0	-1	3947	4567	3937
12	1	0	0	1	4878	4391	4884
13	0	-1	-1	0	1947	2479	1064
14	0	-1	1	0	5619	6953	5632
15	0	1	-1	0	8000	6985	7933
16	0	1	1	0	9889	9675	9898
17	-1	0	-1	0	2772	3384	2783
18	-1	0	1	0	9889	9948	9886
19	1	0	-1	0	4878	5344	3263
20	1	0	1	0	6030	5943	6038
21	0	-1	0	-1	2975	2888	3676
22	0	-1	0	1	3947	4215	4201
23	0	1	0	-1	8586	8841	8643
24	0	1	0	1	4878	5490	5831
25	0	0	0	0	9216	9215	9193
26	0	0	0	0	9214	9215	9193
27	0	0	0	0	9212	9215	9193

$NT_{RSM\ model}$[1], Nisin titer predicted by RSM model.

U_2U_4 (interaction effect between PEP and NaCl) and all of the quadratic terms were significant in the RSM model.

The twenty-seven Box-Behnken design experimental data were randomly divided into three sets: nineteen of them as calibration set, four as prediction set, and the last four as test set. The ANN model was trained with Levenberg-Marquardt algorithm for 1000 interations. Since D_a achieved the maximum value when there were 15 hidden nodes, the number of hidden nodes was set to 15.

The R^2 of the RSM and ANN models were 0.9394 and 0.9829, respectively, the MS*E* of them were 342222.1 and 206225.7 indicating that the fits of the RSM model and ANN model were satisfied.

The optimized fermentation medium by RSM and ANN-GA

The optimum concentrations of four components in the fermentation medium obtained by RSM model using partial differentiation method were (g/l): GLU 19.3, PEP 28.6, YEP 24.1 and NaCl 5.9. The best solution was expected to result in NT of 10531 IU/ml. The verified experiments were implemented with this fermentation medium in triplicate, and the average NT was 10070 IU/ml. The relative error between the expected value and the verified was 4.3%.

The three best solutions obtained by GA after conducting numerous (~50) generations were: GLU 16.81, 16.91 and 16.01 g/l; PEP 28.59, 28.82 and 28.32 g/l; YEP 27.20, 27.20 and 27.20 g-/l; NaCl 5.25, 5.23 and 5.22 g/l. It was also observed that despite beginning the search in a different search space, the GA converged to similar optimal solution corresponding to the lowest local or global minimum on the fitness function surface. The expected NTs with the GA-optimized solutions were: 13209, 13206 and 13216 IU/ml. The best set of fermentation medium was expected to result in NT of 13216

Table 4. The statistical results of multi-quadratic regression analysis of the Box-Behnken design.

Source	DF	SS	MS	F	p(Pr > F)
U_1	1	3135738	3135738	4.0811	0.0663
U_2	1	39181924	39181924	50.9941	0.0001
U_3	1	38484447	38484447	50.0863	0.0001
U_4	1	3070479	3070479	3.9961	0.0688
U_1U_1	1	13720188	13720188	17.8564	0.0012
U_1U_2	1	1836134	1836134	2.3897	0.1481
U_1U_3	1	8894825	8894825	11.5763	0.0052
U_1U_4	1	699186.1	699186.1	0.9100	0.3589
U_2U_2	1	8140014	8140014	10.5940	0.0069
U_2U_3	1	795025	795025	1.0347	0.3291
U_2U_4	1	5472713	5472713	7.1226	0.0205
U_3U_3	1	11309770	11309770	14.7193	0.0024
U_3U_4	1	502410.7	502410.7	0.6539	0.4345
U_4U_4	1	36629387	36629387	47.6720	0.0001
Model	14	1.4299E8	10213769	13.2929	0.0001
Linear	4	83872587	20968147	27.2894	0.0001
Quadratic	4	40919888	10229972	13.3140	0.0002
Cross Product	6	18200294	3033382	3.9479	0.0206
Error	12	9220348	768362.3		
Lack of fit	10	9220348	922034.8	5.849E12	0.0001
Pure Error	2	2	3.153E-7		
Total	26	1.5221E8			

IU/ml. The verified experiments of each GA-optimized solution were implemented in triplicate and the average NTs were: 13536, 13428 and 13529 IU/ml. The relative error between the expected values and experiments was lower than 0.25%, which was also lower than that obtained by the RSM model. The maximum NT obtained by ANN-GA increased 34.0% from that obtained by the RSM model.

The further Box-Behnken design experiments and ANN-GA optimization results

The optimized concentration of YEP obtained by ANN-GA was 27.20 g/l which is the same as the highest level in the test region. It indicated the possibility that the optimum solution was beyond the test regions. Therefore, a further Box-Behnken design was developed based on the statistical results of RSM model. According to the statistical results, the effect of NaCl on NT was non-significant and its optimized concentration was located at the center of the test regions. The concentration of NaCl was set at 5.25 g/l, which was obtained by the first ANN-GA model in the second Box-Behnken design. As the linear statistical results of RSM model indicated, the effect of GLU on NT was negative, while the effects of PEP and YEP were positive. Therefore, the optimized concentration of GLU from the first ANN model was set at the highest level and the optimized concentration of PEP and YEP was set at the lowest levels. The step changes of real values of the variables were set based on the coefficients of the linear terms in the RSM model. The low (-1), middle (0) and high level (1) of the GLU (Z_1), PEP (Z_2) and YEP (Z_3) for further Box-Behnken design were 11.17, 13.99 and 16.81 g/l; 18.59, 28.59 and 38.59 g/l; 27.20, 37.11 and 47.02 g/l, respectively, and the results are shown in Table 5. The ANN was applied to model the correlation of the test factor and NT. The process of establishing and optimizing ANN model is similar to that of the first ANN model. The fourteen sets of Box-Behnken design experimental data were randomly divided into three categories: Ten of them as calibration set; two as prediction set and two as test set. The ANN model was trained with Levenberg-Marquardt algorithm and the number of interations was 1000. The most suitable number of hidden nodes selected by D_a was 12. GA was employed to search for the optimum solutions in the new tested regions. The parameters of GA were the same as those used in the first ANN model.

The optimum solution obtained by ANN-GA was: GLU 15.920, PEP 30.572, and YEP 39.074 g/l. The optimum solution was expected to result in NT of 22216 IU/ml. Three validation experiments with the optimum solution were carried out and the average NT was 21423 IU/ml. The relative error between experimental and expected value was 3.6%.

Table 5. The further Box-Behnken design matrix and experimental results.

Run	Z_1	Z_2	Z_3	Experimental NT (IU/ml)	$NT_{ANN\ model}$ (IU/ml)
1	-1	-1	0	15280	14672
2	-1	1	0	12361	12355
3	1	-1	0	14238	14241
4	1	1	0	20270	18654
5	0	-1	-1	5295	5746
6	0	-1	1	16398	16396
7	0	1	-1	18887	17325
8	0	1	1	18887	18879
9	-1	0	-1	4934	4938
10	1	0	-1	15280	15278
11	-1	0	1	10733	10732
12	1	0	1	15280	15292
13	0	0	0	16398	15837
14	0	0	0	15280	15837

DISCUSSION

L. lactis subsp. lactis is a well-known nutritionally fastidious microorganism requiring an abundance of nutrients for cell growth and metabolism (Kim et al., 1997). The results of the PBD data shows that the effect of GLU, PEP and YEP was significant (namely, the carbon sour-ces and the nitrogen sources in the fermentation medium are the key components). The effect of carbon sources and nitrogen sources is positive, indicating that the initial concentrations were too low. Appropriately increasing the concentrations of carbon and nitrogen sources will elevate the biomass and enhance nisin production. It was reported that the abundant amino acids such as serine, threonine and cysteine in PEP and YEP highly stimulated nisin production (De Vuyst, 1995), which explains why nisin production can be elevated by increasing the concentrations of PEP and YEP. PSA was employed to search appropriate concentrations of the significant components.

The R^2 of the RSM model and ANN model were 0.9394 and 0.9829 respectively, indicating that the fits of the RSM model and ANN model were satisfied. The fit of ANN model were much better than those of the RSM model. The relative error between the expected value obtained by RSM model and the verified was 4.3%. The relative error between the expected value obtained by ANN-GA and the verified was 0.25%. These results demonstrated that the predictive capability of ANN model was much better than that of the RSM model. These results agreed with the conclusion of several literatures (Desai et al., 2008; Kasiri et al., 2008; Pal and Vaidya, 2009). The maximum NT obtained by ANN-GA increased by 34.0% from that obtained by RSM model. These results demonstrated that the true optimum fermentation medium would not be obtained without ANN-GA. The response surface and contours of RSM model and ANN model, respectively, are shown in Figure 1. As can be seen, the response surface obtained by RSM model was convex and their contours were regular. However, the response surface and contours obtained by ANN model seem to be much more nonlinear and complex. The shapes of contours indicated the mutual interaction effects between the test factors. If the shape of the contour is elliptical, the mutual interaction between the two factors is significant; otherwise, if it is circular, the mutual interaction effect is non-significant. From the elliptical contour in Figure 1a, the mutual interaction effect between GLU and YEP was significant. It can also be concluded that the effect of the YEP with low GLU was much more significant than that with high GLU (namely, GLU inhibit YEP for stimulating nisin product). This phenomenon may be caused by the concentration of GLU being excessive than that needed for cell growth which could lead to the accumulation of the excessive acid metabolites such as lactate which inhibit nisin production (Wardani, et al., 2006). As Figure 1b shows, the optimum regions in the response surface and contours of the ANN model were located at the verge of the test regions. It is possible that the optimum fermentation medium for nisin production was beyond the test regions. A further Box-Behnken design experiments were implemented based on the statistical results of RSM and ANN-GA model, and then a new ANN model was developed. GA was employed to search for the optimum solution. The obtained optimum solution was: GLU 15.92, PEP 30.57, YEP 39.07, NaCl 5.25, K_2HPO_4 10, and $MgSO_4 \cdot 7H_2O$ 0.2 g/l with predicted NT of 22216 IU/ml. When compared to the optimum fermentation medium obtained by the first ANN model, the concentrations of PEP and YEP in this fermentation medium increased to 7.95 and 43.62%, respectively. The predicted NT increased to 823%. These results indicated that PEP and YEP can highly stimulate nisin production and dramatically enhance the yield of nisin when the GLU

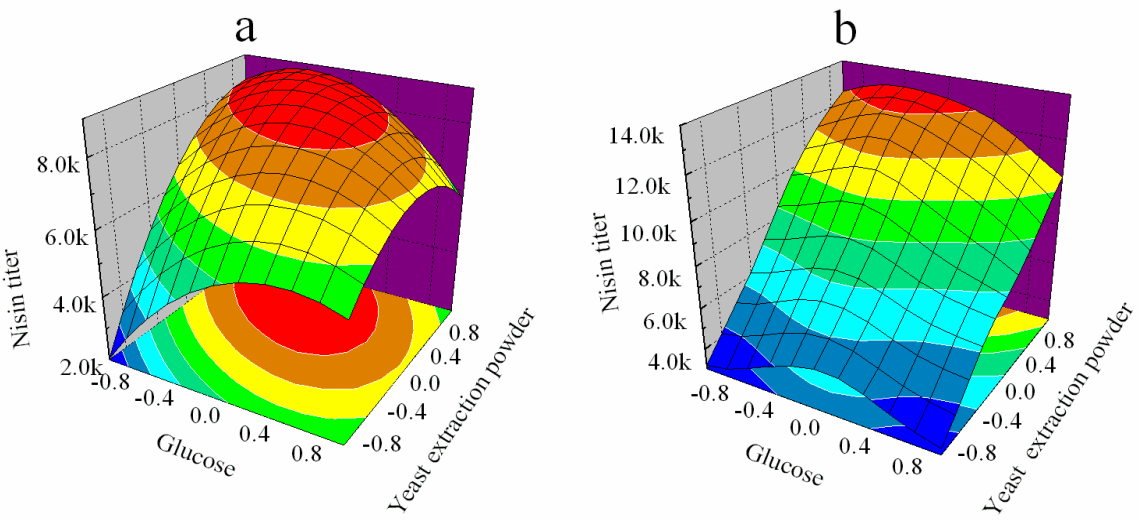

Figure 1. Response surface and contours of the GLU and YEP obtained by RSM model and ANN model (a: RSM model; b: ANN model).

was appropriate. The validation experiments with the optimum solution were implemented in triplicate and the average NT was 21423 IU/ml, which was 8.34 times higher than that without optimization. The relative error between the experimental and expected value was 3.6%, which indicated that the proposed method is feasible.

Abbreviations

RSM, Response surface methodology; **ANN**, artificial neural network; **GA**, genetic algorithm; **PBD,** Plackett-Burman design; **PSA,** path of steepest ascent method; **NT,** nisin titer; **GLU,** glucose; **PEP,** peptone; **YEP,** yeast extraction powder; **EDTA,** ethylenediaminetetraacetic acid; **DES,** diethyl sulfate; **MLP,** multilayer perceptron.

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Full Length Research Paper

Genetic analysis of fertility restoration genes for WA-type cytoplasmic male sterility in Iranian restorer rice line DN-33-18

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Cytoplasmic genetic male sterility system can be used to exploit heterosis in grain crops only when the effective restorer lines are available. Crosses between an Iranian CMS line Neda A with three newly developed lines DN-33-1, DN-33-18 and DN-32-6 showed that only the cross of Neda-A/DN-33-18 had more than 80% pollen and grain fertility in F_1 generation. The inheritance of fertility restoration in F_2 population of this cross was evaluated at flowering and grain filling stages. Pollen staining test with 1% I_2KI solution showed segregation ratio of 15:1 (fertile: sterile), representing two nuclear independent dominant genes controlling the trait carried by fertile parent DN-33-18. Segregation for spikelet fertility in F_2 confirmed the results of pollen fertility test. Molecular tagging of fertility restorer genes with SSR markers showed that four markers RM258, RM171, RM591 and RM3148 produced polymorphic bands between two parents. Linkage analysis on F_2 recessive class showed that RM258 and RM171 (on chromosome 10) flanked to restorer gene *Rf4* at the distances of 3.1 and 6.3 cm, respectively. In this study polymorphism was not detected for *Rf3* gene using SSR markers RM1, RM443, RM315 and RM294 on chromosome 1 of rice but we found new SSR marker RM3148 linked with *Rf* locus at a genetic distance of 19.7 cm.

Key words: Rice, cytoplasmic male sterility (CMS), inheritance, molecular tagging, simple sequence repeat (SSR) markers.

INTRODUCTION

Plant cytoplasmic male sterility (CMS) caused by lesion or rearrangement of mitochondrial genome is unable to produce functional pollens. But CMS can be restored by nuclear genes. Therefore, the CMS systems are widely used for hybrid seed production. In rice, more than 90% of the rice hybrids developed over these years belongs to wild abortive cytoplasmic source (Yao et al., 1997).

Hybrid breeding based on CMS/*Rf* system has achieved great success all over the world. Three primary types of CMS in rice are known, and their heritance habits and physiological characteristics have been extensively investigated. They are Wild-rice abortive (WA), BaoTai (BT) and HongLian (HL). WA type CMS belongs to sporophytic abortion, which fails to produce normal pollen and finally forms typical abortive pollen. In contrast, BT (*japonica*) and HL type CMS (*indica*) belong to gametic abortion, but they are also greatly different in terms of abortive phenotype, relationship of restoration, and maintenance (Sattari et al., 2008).

BT type CMS is restored by nuclear fertility restorer gene *Rf1*, which was mapped on chromosome 10 (Shinjio, 1975; Fukuta et al., 1992; Akagi et al., 1996; Yokozeki et al., 1996). HL type fertility restoration gene *Rf5* was also mapped on chromosome 10 (Huang et al., 2003). The inheritance of fertility restoration in WA-CMS system has been extensively investigated. Using RFLP markers, Zhang et al. (1997) mapped one of the two *Rf* loci (*Rf3*) on chromosome 1 between RG140 and RG532

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Abbreviations: CMS, Cytoplasmic male sterility; **WA,** wild-rice abortive; **BT,** BaoTai; **HL,** HongLian; **RAPD,** randomly amplified polymorphic DNA; **RFLP,** restriction fragment length polymorphism; **MAS,** marker assisted selection; **SSR,** simple sequence repeat; **NILs,** near isogenic lines.

Table 1. List of the used SSR primer pairs with their chromosomal locations and annealing temperature.

SSR marker	Chromosome	Forward primer	Reverse primer	Annealing temperature (°C)
RM1	1	gcg aaa aca caa tgc aaa aa	gcg ttg gtt gga cct gac	55
RM443	1	ggg agt tag ggt ttt gga gc	tcc agt ttc aca ctg ctt cg	55
RM315	1	cgg tca aat cat cac ctg ac	caa ggc ttg caa ggg aag	55
RM294	1	ttg gcc tag tgc ctc caa tc	gag ggt aca act tag gac gca	55
RM6344	7	aca cgc cat gga tga tga c	tgg cat cat cac ttc ctc ac	55
RM171	10	aac gcg agg aca cgt act tac	acg aga tac gta cgc ctt tg	67
RM258	10	tgc tgt atg tag ctc gca cc	tgg cct tta aag ctg tcg c	55
RM244	10	ccg act gtt cgt cct tat ca	ctg ctc tcg ggt gaa cgt	55
RM591	10	cgg tta atg tca tct gat tgg	ttc gag atc caa gac tga cc	55
RM3123	10	att tcc cac aca tct cgc tg	gtg tcg ccg gtc aag aac	55
RM7003	12	ggc aga cat aca gct tat agc	tgc aaa tga acc cct cta gc	55

at a distance of 1.9 cm from each. Using RAPD and RFLP markers, Yao et al. (1997) confirmed the location of *Rf3* on chromosome 1 and mapped the second *Rf* locus (Rf_4) on chromosome 10 at 3.3 cm from G4003. Jing et al. (2001) mapped an *Rf* locus (Rf_4) governing fertility restoration on the long arm of chromosome 10 using SSLP markers. Zhang et al. (2002) also mapped the *Rf4* gene on chromosome 10 at 0.9 cm from the marker Y3-8 and anchored to the RFLP marker S10019. Bazrkar et al. (2008) tagged four *Rf* genes for WA –CMS system using SSR markers on chromosomes 1 (*Rf3*), 7 (*Rf4*), 10 (*Rf6*) and 12 (*Rf7*) by recessive class analysis.

The most straightforward method of marker identification would be finding different banding patterns in two sets of lines representing contrasting phenotypic classes. The main precondition for starting molecular analysis aimed at finding useful markers for a given trait is a basic knowledge of its inheritance. This is usually gained through classical population genetic analysis. Recognition of the gene or genes underlying the trait under study opens the possibility of looking for linked molecular markers. Detection of segregation mode of fertility restoring genes in newly developed Iranian *indica* rice DN-33-18 and identifying molecular markers linked to that trait for use in marker assisted selection (MAS) was the aims of this study.

MATERIALS AND METHODS

Plant materials and population development

In this study an Iranian CMS-WA line Neda A, with newly developed cultivars DN-33-1, DN-33-18 and DN-32-6 were crossed in 2007 and F_1 seeds were obtained. Neda A has been improved through backcross method using IR58025A from IRRI (Nematzadeh et al., 2006). DN-33-1, DN-33-18 and DN-32-6 are advanced lines under release in north of Iran, Mazandaran province which improved through pedigree-backcross method from the cross between Sepidroud and Sang Jo. Only the cross of Neda-A / DN-33-18 had more than 80% pollen and grain fertility in F_1 generation. So, the inheritance of pollen fertility restoration in F_2 population of this cross including 328 individual plants was evaluated at flowering and grain filling stages.

Evaluation of pollen and spikelet fertility

In pollen fertility test, 3 panicles per plant were selected to prepare pollen samples and 1% I2-KI solution was used to identify fertile and sterile pollens. Pollen fertility was evaluated by shape, size and stained color: Fertile-grain with morphologically spherical and darkly stained, and sterile-grain with spherical but small and stained in light brown. In spikelet fertility test, 3 panicles per plant were bagged with paper pocket to prevent outcrosses and percentage of fertile spikelets was calculated. X^2 test was used to test fitness of observed genetic ratio with expected ratio in F_2 population.

DNA extraction and PCR analysis

Total genomic DNA was extracted according to Dellaporta et al. (1983). PCR amplification was performed using the DNAs from parental lines Neda A and DN-33-18 along with completely sterile plants from F_2 population of Neda A/DN-33-18. The DNAs were subjected to the amplification by 11 SSR markers (Table 1). A 25 µl mixture was prepared for the PCR assay containing 50 ng template DNA, 2.5 µl of 10X buffer, 0.3 µl of 10 mM dNTPs, 1 µl of 50 mM $MgCl_2$, 1 µl of each primer, and 1 unit of *Taq* polymerase. The PCR reaction was performed at 94°C for 5 min (initial denaturation); then for 35 cycles of 94°C for 1 min; 50 - 67°C for 1 min; 72°C for 2 min followed by 72°C for 5 min. PCR products were resolved by electrophoresis in 3.5% agarose gel containing 0.5 µg/ml ethidium bromide.

Linkage analysis

Map distances were based on the Kosambi function (Kosambi, 1944). Linkage groups were assigned to corresponding chromosomes based on SSR markers mapped by McCouch et al. (2002). For single-marker analysis, the recombination frequency between a positive marker and an *Rf* locus was calculated using maximum likelihood estimator (Allard, 1956), assuming that all the extremely sterile individuals were homozygous at the targeted *Rf* locus.

Table 2. Testcross of advanced lines with Neda A for determination of their ability to restoration of fertility in CMS-WA system.

Cross	Pollen fertility	Spikelet fertility	Ability to restoration of fertility
NedaA/ DN-33-1	<80	<80	Partial restorer
NedaA/ DN-33-18	>80	>80	Restorer
NedaA/ DN-32-6	<80	<80	Partial restorer

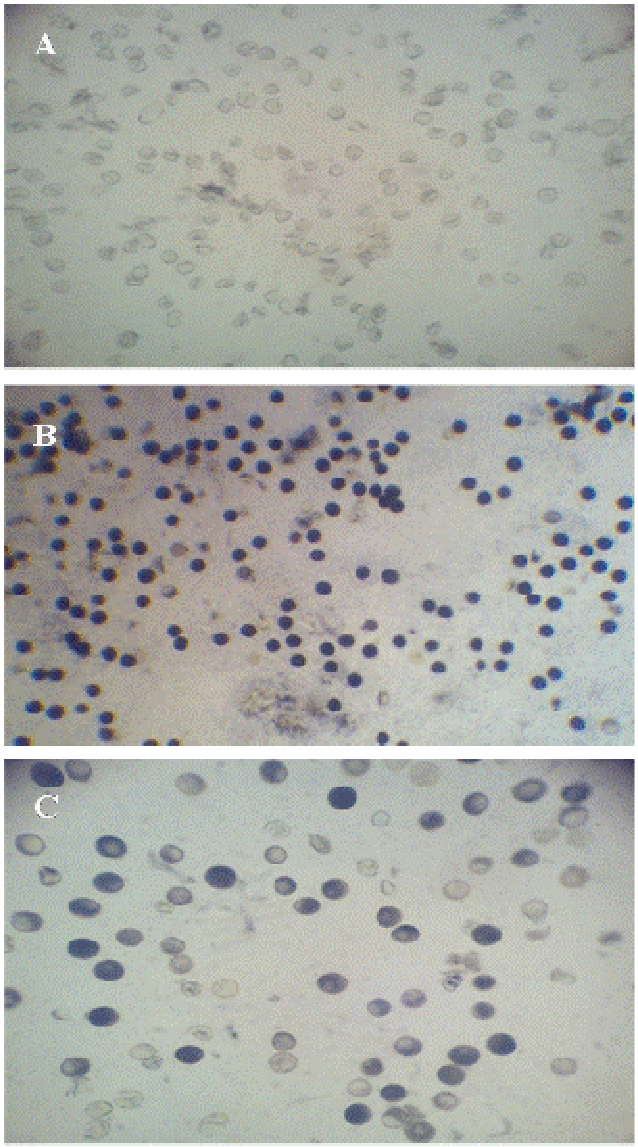

Figure 1. Segregation of pollen fertility in F_2 population of NedaA/DN-33-18 using 1% I_2KI solution. Completely sterile plants (A) vs. completely and partial fertile plants (B, C).

RESULTS

Segregation of fertility restoration

Taking seed setting rate as fertility criterion, the F_1 Neda A/DN-33-18 showed more than 80% fertility (Table 2) so this cross was chosen to study of genetic bases of fertility restoration for Neda A with WA type of sterile cytoplasm. Lines DN-33-1 and DN-32-6 showing partial fertility in test cross with CMS line Neda A.

The pollen fertility test in F_2 population of Neda A/DN-33-18 revealed segregation into 312 fertile and 16 completely sterile plants (Figure 1), indicating that the expected 15 : 1 (fertile: sterile) ratio is good (Table 3).

Detection of fertility restoration genes in DN-33-18

Eleven microsatellite primers that were reported to link with fertility restoring genes in different chromosomal locations (chromosomes 1, 7, 10 and 12) were screened for polymorphism between the parents. Microsatellite primers RM171, RM258 and RM591 all on chromosome 10 of rice produced polymorphic products. Then these primers were used in linkage analysis with 16 completely sterile plants from F_2 population of NedaA/DN-33-18. Linkage analysis on recessive class showed that RM258 and RM171 closely linked to restorer gene *Rf4* at the intervals of 3.1 and 6.3 cm from it (Table 4). In this study, analysis of SSR markers for *Rf3* on chromosome 1 shows no polymorphism using RM1, RM443, RM315 and RM294 markers. Extra analysis with other SSR markers achieved for detection of chromosomal location of second *Rf* gene in Iranian restorer line DN-33-18. We detected new SSR marker RM3148 on chromosome 1 (Figure 2) linked with second *Rf* gene at a genetic distance of 19.7 cm in line DN-33-18 (Figure 3).

DISCUSSION

Classical analysis of fertility restoring genes showed that fertility restoration for the Neda A with WA type of cytoplasm is controlled by two dominant genes carried by Iranian *indica* line DN-33-18. The study result is in accordance with several studies on *indica* rice (Zhou, 1983; Young and Virmani, 1984; Govinda Raj and Virmani, 1988; Teng and Shen, 1994).

This study, successfully tagged two *Rf* genes in restorer line DN-33-18 for WA type of CMS system. One *Rf* gene on rice chromosome 10 flanked by RM258 and RM171 markers. Sattari et al. (2008) clarified that *Rf* genes for WA-, HL- and BT-type CMS restoration systems were located on chromosome 10, and this *Rf*

Table 3. Distribution for pollen and spikelet fertility (numbers in parentheses) in parents, F_1 and F_2 population of the cross Neda A/DN-33-18.

Progeny	Fertile	Completely sterile	Genetic ratio	$\chi^{2\,a}$
Neda A	-	10		
DN-33-18	10	-		
F_1	10	-		
F_2	312 (316)	16 (12)	15: 1	X^2=1.054 ^{ns} (3.760 ^{ns})

[a] Critical χ^2 for 1 degree of freedom is 3.84 (p = 0.05).

ns: Not significant at 5% statistical level.

Table 4. Recombination frequencies and genetic distances between the positive markers and the *Rf* locus calculated using Maximum likelihood method, based on the assumption that all the extremely sterile plants are homozygous for the recessive allele at targeted loci.

Locus	Chromosome	Recombination frequency (%)	Genetic distance	LOD score
RM258	10	3.12	3.13	3.85
RM171	10	6.25	6.28	3.19
RM591	10	37.5	37.95	0.22
RM3148	1	18.75	19.71	1.46

LOD, Limit of detection.

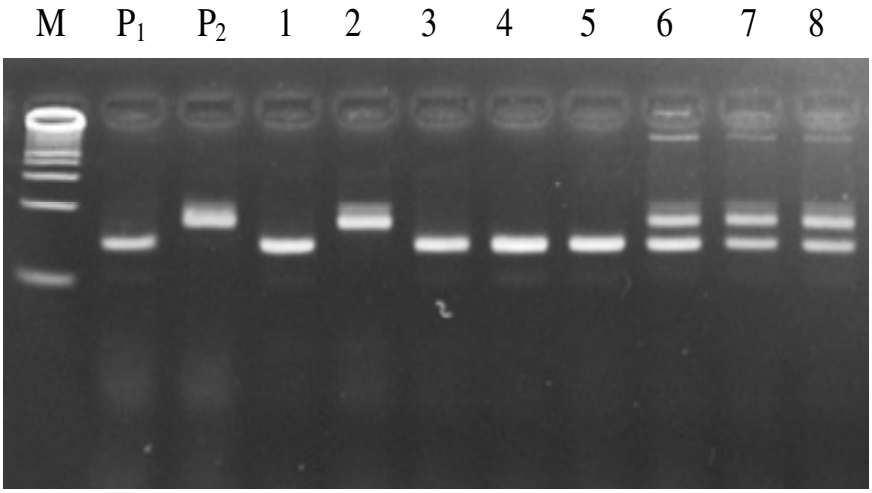

Figure 2. Linkage analysis on sterile plants from F_2 population of Neda A/DN-33-18 using SSR marker RM3148 located on chromosome 1 of rice. M: 100bp ladder, P_1: Neda A, P_2: DN-33-18 and numbers 1 to 8 are some of individual sterile plants.

gene that was detected is located at the same region. The second *Rf* gene located on chromosome 1 of rice was not linked with SSR markers that was reported to be link with *Rf3* gene but this study found a new marker RM3148 on short arm of chromosome 1 at a distance of 19.7 cm. Therefore, the inheritance mechanism and fine mapping of this gene and its relation with *Rf3* is yet to be elucidated.

Marker assisted selection (MAS) is being explored as an important supplement to phenotypic selection in rice breeding. PCR based markers offers great potential to enhance the efficiency of MAS. SSR markers have the advantages of rapidity, straight, and simplicity of RAPD, and the stability, reliability, and repeatability of RFLP. The results presented here clearly indicate that the micro-satellite markers RM258, RM171 and RM3148 will be facilitating MAS of restorer lines in CMS-WA system from large source nurseries to avoid routine testcrosses in hybrid breeding programs. It is also expected that the use of these microsatellite markers in MAS integrated with

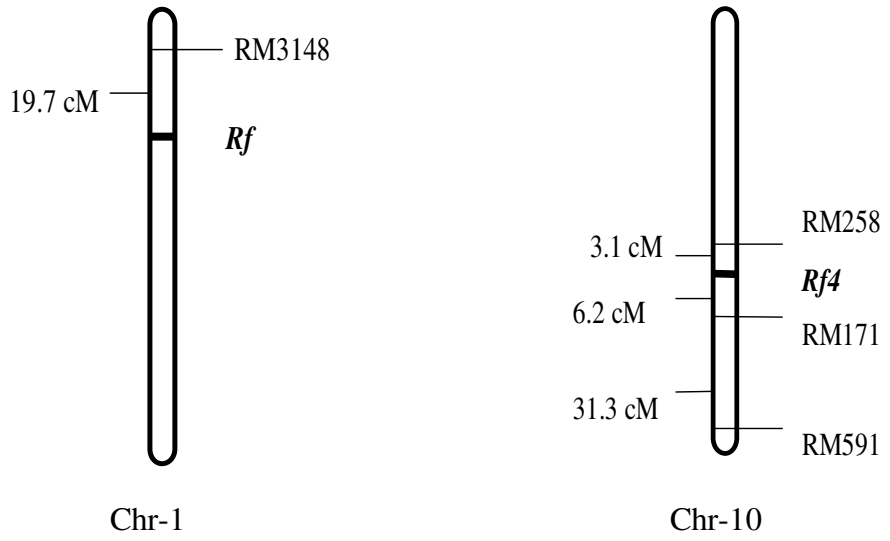

Figure 3. Linked SSR markers with fertility restoring genes on chromosomes 1 (left) and 10 (right) of rice.

backcross breeding will produce near isogenic lines (NILs) of fertility restorer lines for genetic research.

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Full Length Research Paper

Study of fecal bacterial diversity in Yunnan snub-nosed monkey (*Rhinopithecus bieti*) using phylogenetic analysis of cloned 16S rRNA gene sequences

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The bacterial diversity in fecal samples from Yunnan snub-nosed monkey (*Rhinopithecus bieti*) was investigated by constructed 16S rRNA gene clone library and restriction fragment length polymorphism analysis. As a result, a total of 156 representative clones for each profile, comprising nearly full length sequences (with a mean length of 1.5 kb) were sequenced and submitted to an on-line similarity search and neighbor-joining phylogenetic analysis. Using the criterion of 97%, these 16S rRNA gene sequences were binned in 129 OTUs. 11 sequences whose similarity is ≥ 97% were affiliated to the cultured bacteria and accounted for 7.05% of the total clones. For 23 sequences (14.74%), the similarity with the database was in the range of 89 - 97%. The remaining 122 sequences (78.21%) were uncultured and unidentified bacteria. Based on the phylogenetic analysis, the fecal bacteria of *R. bieti* distributed mainly in 6 bacteriophyta of *Firmicutes, Proteobacteria, Bacteroidetes, Fibrobacteres, Spirochaetes* and *Actinobacteria,* and belonged to 17 genera. Besides, there were a large number of uncultured and unidentified bacteria. These results illustrate the fecal bacteria diversity of *R. bieti*.

Key Words: *Rhinopithecus bieti*, fecal bacterial diversity, 16S rRNA gene, phylogenetic analysis.

INTRODUCTION

Alias Yunnan snub-nosed monkey or black snub-nosed monkey (*Rhinopithecus bieti*) belongs to *Rhinopithecus, Colobinae, Cercopithecidae,* and *Primates* (Li and Lian, 2007). *R. bieti* is one of the national first-class protected animals and a world-class rare species (Bai et al., 1988), which had been listed among the highly endangered species by the International Union on Conservation of Nature (IUCN) (Reng et al., 2004). The existing natural population of this species is about 1,500 in 13 species groups, which distribute along the narrow terrain between Jingsha River in the east, the eastern bank of Lancang River in the west, Yunlong County of Yunnan Province in the south, and Markam County of Tibetan Autonomous Region of Tibet in the north, China (Long et al., 1996).

The stomach of *R. bieti* is an S-shaped structure (Chen et al., 1995), and consists of the cardiac area, fundus ventriculi+, corpus ventriculi, canalis ventriculi and pylorus. The fundus ventriculi and corpus ventriculi retain a large number of bacteria, which have a similar fermentation function to those in the ruminant rumen, and the pylorus is the area for digestion (Peng et al., 1983). *R. bieti* is the primate who is living on the highest altitude besides human (Long et al., 1996; Zhao, 1998). Unlike other primates, *R. bieti* eats such epiphytes of spruce and fir as usnea, mosses and lichens, and the leaves, flowers and fruits of *Rosaceae, Aceraceae* and other broadleaved trees

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Abbreviations: OUT, Operational taxonomic unit; **IPTG,** isopropyl-β-D-thiogalactopyranoside; **X-Gal,** 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; **LB,** Luria–Bertani; **NCBI,** National Centre for Biotechnology Information; **PCR,** polymerase chain reaction.

as its diet (Zhang et al., 2005). As an herbivorous animal, the feeding habits of *R. bieti* have obvious seasonal variations (Ding, 2003). So far, no other wild animal had been found eating usnea. Such a particular feeding habit with crude fiber foods might be relevant to the special structure of the stomach and intestinal microorganisms. Currently, researches at home and abroad on *R. bieti* has mostly focused on aspects of taxonomy, ecology, anatomy and conservation genetics, but research on fecal bacterial diversity has not been reported.

The large number of microorganisms in the intestine of *R. bieti*, which constitute a complex and dynamic equilibrium of microflora, play an important role in helping the parasitifer digest food and resisting invasion by alien flora (Hooper et al., 2001; Neish et al., 2002). Microbial disequilibrium may lead to physiological dysfunction and diseases (Feng et al., 2005). Traditional classification methods that are based upon purification and culture of the intestinal microbial community are only effective with culturable microorganisms, which means that those intestinal microorganisms that can not be cultured inevitably are ignored (Kocherginskaya et al., 2001). Using traditional research methods, investigations of microbial morphology are so subjective that there are some deficiencies. Moreover, molecular biology studies in recent decades have shown that microbial purification and culture cannot be accomplished for more than 99% of environmental microorganisms (Pace, 1997).

To achieve a comprehensive understanding of the constitution of the intestinal bacterial community of *R. bieti*, we use phylogenetic analysis of cloned 16S rRNA gene sequences. This approach allowed us to establish the diversity of intestinal bacteria in *R. bieti*. Our results may provide a basis for further study of the relationships between the composition of the intestinal microecosystem, the feeding habits of *R. bieti* and the structure of the bacterial communities. They also may lay a foundation for the development and utilization of microbial resources, and for the conservation of this wild animal.

MATERIALS AND METHODS

Experimental material

100 samples of *R. bieti* feces were collected in November 2008 from Longma Mountain in Yunlong County of Dali Prefecture, Yunnan Province, China. Fecal samples (taken ≤12 h after defecation) were collected from 80 - 120 *R. bieti* in a single group with the help of the experts of *R. bieti*, who can distinguish these monkeys' feces from the feces of other animals. Samples of fresh material were soaked in 100% ethanol (Li et al., 2006), transported to the laboratory in an icebox, and stored at –70°C (All research reported in this manuscript complied with animal care regulation, applicable national laws, and the ASP Principles for the Ethical Treatment of Non Human Primates).

Total DNA extraction

To minimize animal-to-animal variations, the aliquots of feces from 100 samples were mixed before DNA extraction. Total DNA was extracted using Bacterial Genomic DNA Extraction Kit (TaKara, Dalian, China). The DNA concentration and its integrity (size > 15 kb) were estimated by agarose gel electrophoresis with 0.8% (w/v) agarose, 1× Tris–borate–EDTA buffer, and 1 ng/mL Gelview (Suau et al., 1999).

16S rRNA gene amplification

Polymerase chain reaction (PCR) amplification of bacterial 16S rRNA gene was performed using the universal primers F27 (5′-AGATTGATCMTGGCTAGGGA-3′) and R1492 (5′-TACGGYTA CCTTGTTACGACTT-3′) (Lane, 1991). Samples were amplified in five replicate reactions to minimize stochastic PCR bias (Polz and Cavanaugh, 1998). The PCR was set up in a 50 µL volume that contained 1.5 µL fecal DNA, 5 µL 10× PCR buffer (25 mmol/L Mg^{2+}), 4 µL dNTP (2.5 mmol/µL), 1.5 µL each primer (10 pmol/µL), 0.5 µL Taq DNA polymerase (TaKara Inc. China) and 36 µL ddH_2O. The amplification conditions were as follows: 4 min of initial denaturation at 94°C; followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min 30 s, extension at 72°C for 2 min 30 s, with the last cycle followed by a 20 min extension step at 72°C. The PCR products were combined and visualized on an agarose gel, the bands were excised, and DNA was purified from the gel slices using TaKaRa Agarose Gel DNA Purification Kit. All purified DNA samples were mixed in an eppendorf tube.

Construction of 16S rRNA gene clone library

The purified DNA products were ligated into pMD18T-vectors using a rapid ligation kit according to the instructions of the manufacturer (TaKara Inc. China), and then transformed into competent *Escherichia coli* DH5α cells by heat shock (45 s at 42°C). Clone libraries were established on Luria–Bertani (LB) agar plates with ampicillin (100 µg/ml) and also with IPTG (isopropyl-β-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Colonies that contained a plasmid with an insert could not produce β-galactosidase and degrade X-Gal; consequently, they were white (Suau et al., 1999; Li et al., 2007).

RFLP analysis of 16S rRNA gene

To screen the clones for grouping into similar types, those that contained target 16S rRNA gene were subjected to RFLP analysis. Two vector primers: M13-47 (5′-CGCCAGGGTTTTCCCAGT CACGAC-3′), and RV-M (5′-AGCGGATAACAATTTCACACAGG-3′) (Li et al., 2007) were used to amplify each insert by 30 cycle colony PCR. The products were digested with 1 U each of restriction nucleases *Afa*I and *Msp*I for 6 – 12 h at 37°C. The digested fragments were visualized on a 2.5% agarose gel, and different clones were distinguished according to their RFLP patterns. Clones that produced the same RFLP pattern (DNA fragments of the same size) were grouped together and considered representative of the same operational taxonomic unit (OTU). One clone of each RFLP type was sequenced. The genetic diversity identified by RFLP analysis was subjected to statistical analysis of the percentage of coverage of the library, using the formula (1–(n/N)) ×100% (Good, 1953). Here, n represents the number of clones that occurred only once (16S rRNA gene sequence similarity of < 97%), and N is the total number of clones examined.

Sequencing and sequence analysis

Selected cells were grown overnight in ampicillin-selective LB broth.

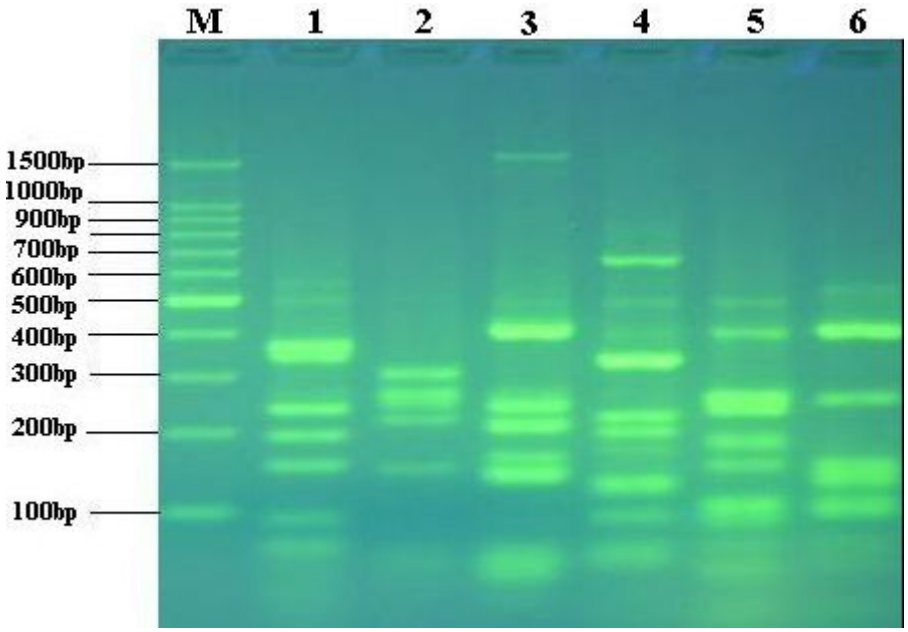

Figure 1. *Afa*I and *Msp*I restriction patterns of 16S rRNA geneM, 100-bp ladder marker; 1– 6, different restriction patterns out of the 156 different patterns.

The clones were sequenced at the Beijing Genomics Institute. The resulting rRNA gene partial sequences were compared with GenBank entries using BLAST to select reference sequences and obtain a preliminary phylogenetic affiliation of the clones. All the sequences obtained were checked for chimeric artifacts using the CHIMERA_CHECK program (Cole et al., 2005). The nearest neighbors were retrieved from NCBI through a BLAST search (http://www.ncbi.nih.gov/BLAST). The most similar sequences were retrieved and aligned with those obtained in this study using Clustal W (Thompson et al., 1994), and the alignments for which homology of residues could not be assumed reasonably were excluded from the phylogenetic analysis. The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with the two-parameter model of Kimura (Kimura, 1980). The bootstrap analysis (Felsenstein, 1985) was based on 1000 resamplings.

Nucleotide sequence accession numbers

The sequences from this study were submitted to GenBank under accession numbers GQ451171–GQ451325.

RESULTS

RFLP analysis of the fecal bacterial population

The colony PCR products showed different types of bands after digestion with the restriction nucleases *Afa*I and *Msp*I (Figure 1). At last, we got a total of 156 RFLP groups, which allowed for small variations. Although the RFLP results can not reflect accurately all intestinal microbial groups present in *R. bieti*, they do reflect the richness of the library. Each type was grouped into an operational taxonomic unit (OTU). There were some differences between clones that grouped are within an OTU; however, they are approximated to a phylogenetic taxonomic group (Moyer et al., 1996). Thus, the coverage calculated was 82.69%, which suggested that this study identified almost the whole dominant biodiversity in the given environment. One clone of each RFLP type was sequenced, with which a mean sequence length of 1.5kb. The results of the check for chimeras showed that all of the sequences obtained were normal.

Cloned sequence alignment analysis

We used a 97% level of sequence identity to define the OTUs (Janda and Abbott, 2002). Clones with similarity > 97% were classified into an OTU. 129 OTUs were identified from the 156 cloned sequences (Table 1). The sequence assignment showed that the highest and lowest similarity to those in Genbank was 99 and 84%, respectively.

Of the 156 clones isolated, 40 (comprising 37 OTUs and represent 25.64% of the clones) had ≥ 97% similarity of sequences with known species of bacteria, which included *Pseudomonas* sp., *Pedobacter* sp., *Yersinia enterocolitica, Ruminococcus, Deefgea* sp., *Budvicia aquatica, Sphingobacterium* sp., *Pseudomonas syringae, Pelosinus* sp., *Mycetocola saprophilus, Comamonas* sp. and some uncultured bacteria. Another 105 clones (comprising 81 OTUs and representing 67.31% of the clones) shared a sequence similarity of 90 – 97% with known species of bacteria, and the remaining 11 clones (comprising 11 OTUs and representing 7.05% of isolated

Table 1. Distribution of 16S rRNA gene clones and operational taxonomy units (OTU) retrieved from the feces of *Rhinopithecus bieti*

Items	Clones		OTU[a]	
	Number of clones	%Total clones	Number of OTU	%Total OUT
Similarity				
≥ 97%	40	25.64	37	28.68
90 - 97%	105	67.31	81	62.79
< 90%	11	7.05	11	8.53
Phylum				
Firmicutes	33	21.15	31	24.03
Proteobacteria	14	8.97	12	9.30
Bacteroidetes	7	4.49	7	5.43
Fibrobacteria	8	5.13	5	3.88
Spirochaetes	16	10.26	8	6.20
Actinobacteria	6	3.85	6	4.65
Unclassified	72	46.15	60	46.51
Total	156		129	

a: Clones having > 97% similarity of 16S rRNA gene among each other were defined as 1 operational taxonomy unit (OTU).

clones) have less than 90% similarity with sequences of known bacteria (Table 1).

Phylogenetic analysis

The similarity for most of the sequences with those of known bacteria was too low to identify the sequence as representing a particular taxon, therefore, phylogenetic trees were constructed to investigate the taxonomic placement. Phylogenetic trees reveal that the intestinal bacteria, extracted from the feces of *R. bieti*, are plentiful and they mainly distributed among the *Firmicutes, Proteobacteria*, *Bacteroidetes, Fibrobacteres*, *Spirochaetes*, *Actinobacteria* and unclassified bacteria (Figure 2). And different bacteriophyta have different proportion (Table 1).

Phylogenetic analysis of *Firmicutes*

Of the 156 clones, there were 33 (21.15%) situating at the *Firmicutes* of low G+C gram-positive bacterium (Figure 3). Therein, all bacteria belonged to *Clostridium*, which were the dominant bacteria in the intestine of *R. bieti*. The 33 clones (31 OTUs) had high similarity to the bacteria in the feces and rumen of mammals such as oxen, rats, reindeer, gorillas, and humans. To some extent, this is related to the evolutionary process of mammals. The two sequences J153 and J169 had 95% similarity to the unidentified bacteria in the feces of *Bos frontalis*. J132 had 96% similarity to the uncultured *Ruminococcus* from bovine feces. J169 and J137 shared a 100% bootstrap value with the bacteria from the rumen and feces of cows and the galactophore of dairy cows, which showed high evolutional relativity. J346 had 95% similarity to *Clostridium* separated from the galactophore of dairy cows. J83 and J19 had 96% similarity to the uncultured bacteria separated from the methane tank and dunghill. J260 had 97% similarity to the *Pelosinus* sp. They shared equal distance in the phylogenetic tree. J86 clustered with the uncultured bacteria from feces of gorillas, and shared 97% similarity with a bootstrap value of 100%. There was high evolutional relativity between them. Therefore, the intestinal bacteria of primates are probably similar to each other. J133 had 93% similarity to the bacteria separated from the feces of patients with irritable bowel syndrome and shared a 100% bootstrap value, which indicated the stability of the genetic relationship in evolution.

Phylogenetic analysis of *Proteobacteria*

The sequence alignment and phylogenetic tree structure (Figure 4) showed that, in the 16S rRNA gene clone libraries, there were 34 sequences (28 OTUs) (21.79%) that resembled 16S rRNA gene sequences of the identified bacteria, most of which belonged to the *Proteobacteria*. There were 14 (8.97%) from 156 sequences classified into the *Proteobacteria*, which included the four classes of *Proteobacteria*: α, β, γ and ε, and the eight genera: *Rhodobacter*, *Sphingomonas*, *Rhodopseudomonas*, *Deefgea*, *Comanonas*, *Pseudomonas*, *Yersinia*, and *Campylobacter*. The phylogenetic analysis showed that the bacterium was classified into two clusters.

The three classes β, γ and ε were classified as one cluster, including 8 sequences. J30 and J44 respectively had 99 and 98% similarity with *Yersinia enterocolitica* and

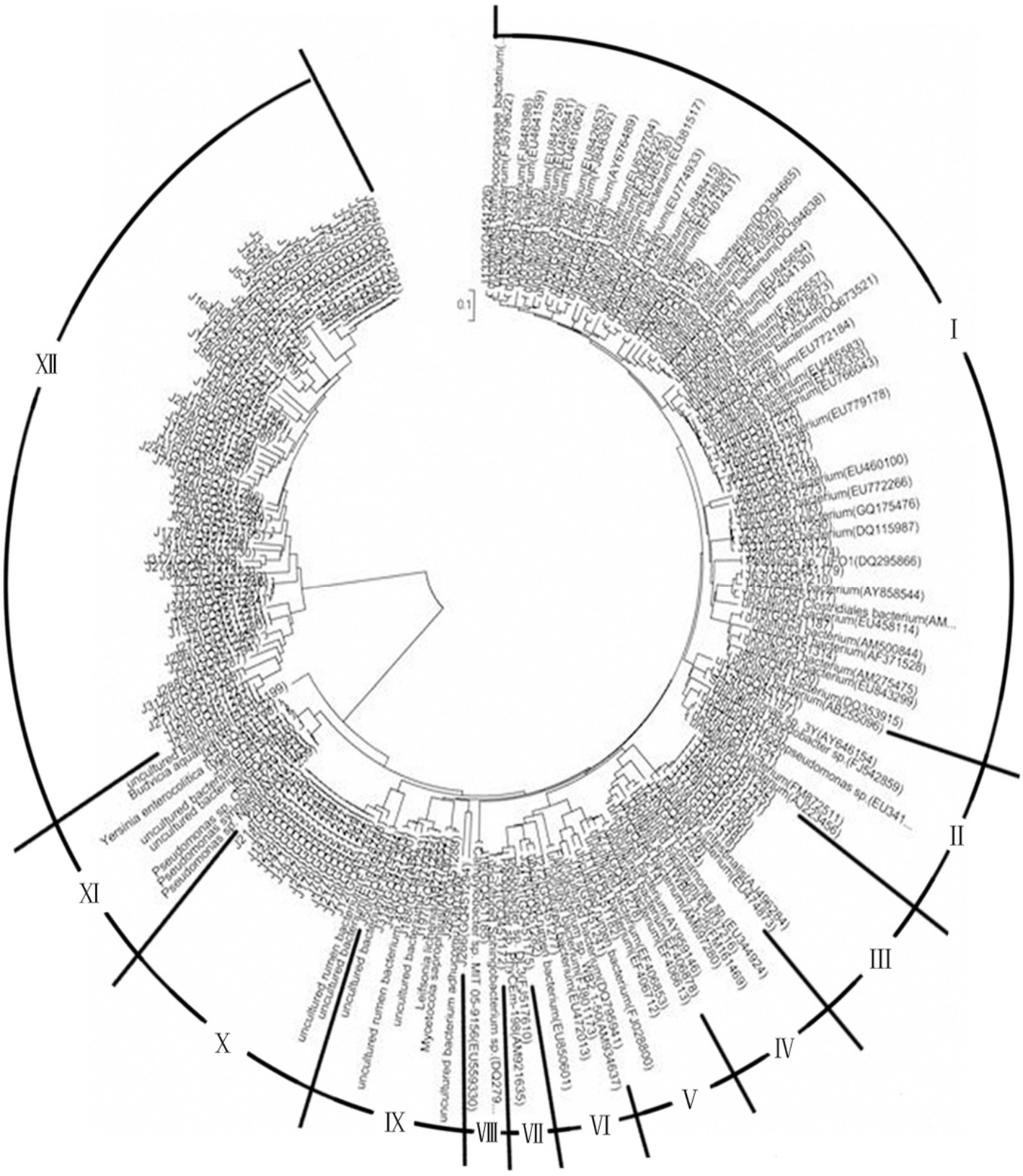

Figure 2. Phylogenetic tree based on 16S rRNA gene sequences from *Rhinopithecus bieti* clones. Numbers in parentheses represent the sequence accession numbers in GeneBank. The bar represents 10% sequence divergence. Roman numerals represent different bacteriophyta.I:*Firmicutes*.II:α-*Proteobacteria*.III:*Fibrobacteres*.IV:β-*Proteobacteria*.V:Bactero-

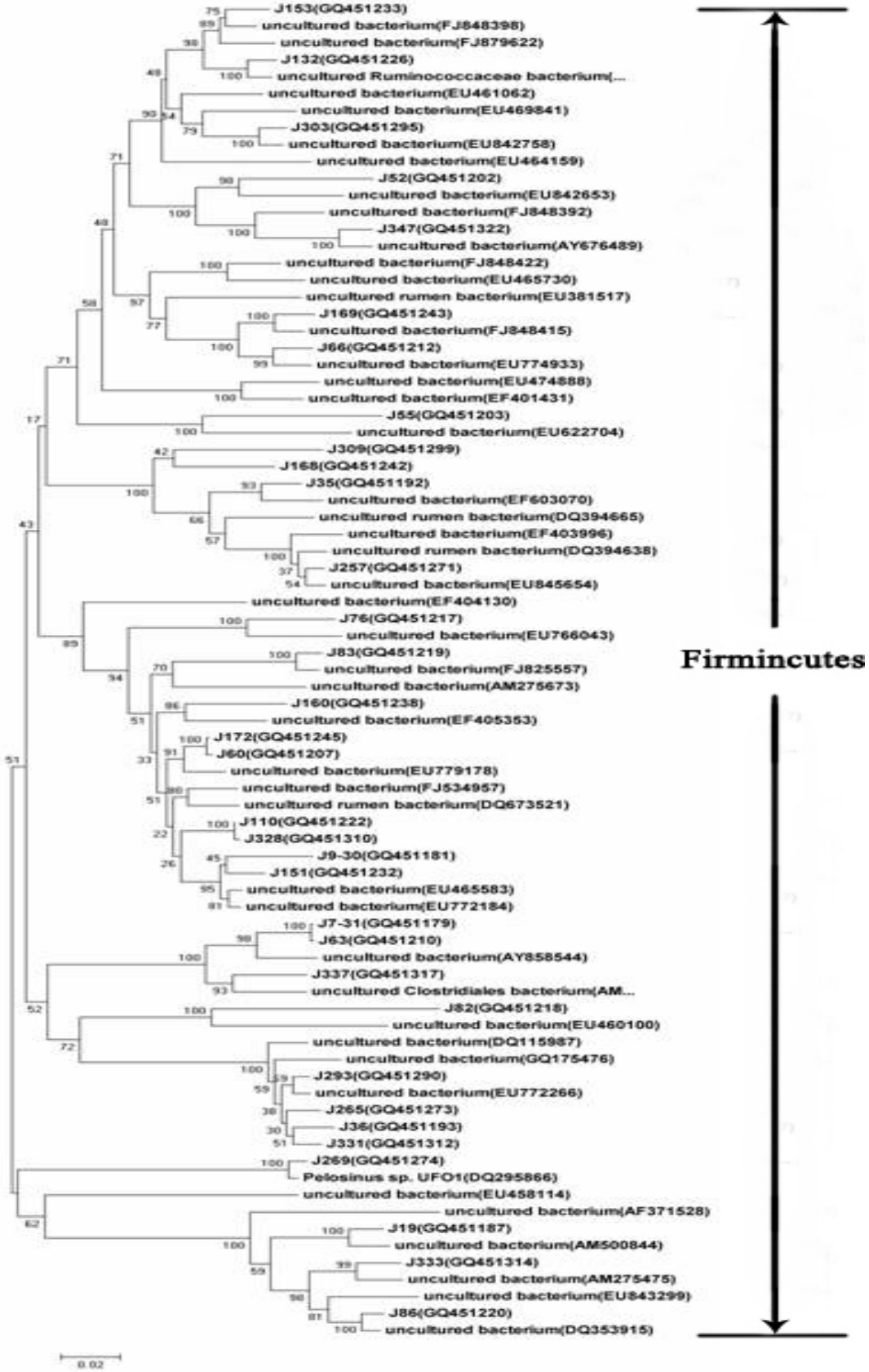
J153(GQ451233)
uncultured bacterium(FJ848398)
uncultured bacterium(FJ879622)
J132(GQ451226)
uncultured Ruminococcaceae bacterium(...
uncultured bacterium(EU461062)
uncultured bacterium(EU469841)
J303(GQ451295)
uncultured bacterium(EU842758)
uncultured bacterium(EU464159)
J52(GQ451202)
uncultured bacterium(EU842653)
uncultured bacterium(FJ848392)
J347(GQ451322)
uncultured bacterium(AY676489)
uncultured bacterium(FJ848422)
uncultured bacterium(EU465730)
uncultured rumen bacterium(EU381517)
J169(GQ451243)
uncultured bacterium(FJ848415)
J66(GQ451212)
uncultured bacterium(EU774933)
uncultured bacterium(EU474888)
uncultured bacterium(EF401431)
J55(GQ451203)
uncultured bacterium(EU622704)
J309(GQ451299)
J168(GQ451242)
J35(GQ451192)
uncultured bacterium(EF603070)
uncultured rumen bacterium(DQ394665)
uncultured bacterium(EF403996)
uncultured rumen bacterium(DQ394638)
J257(GQ451271)
uncultured bacterium(EU845654)
uncultured bacterium(EF404130)
J76(GQ451217)
uncultured bacterium(EU766043)
J83(GQ451219)
uncultured bacterium(FJ825557)
uncultured bacterium(AM275673)
J160(GQ451238)
uncultured bacterium(EF405353)
J172(GQ451245)
J60(GQ451207)
uncultured bacterium(EU779178)
uncultured bacterium(FJ534957)
uncultured rumen bacterium(DQ673521)
J110(GQ451222)
J328(GQ451310)
J9-30(GQ451181)
J151(GQ451232)
uncultured bacterium(EU465583)
uncultured bacterium(EU772184)
J7-31(GQ451179)
J63(GQ451210)
uncultured bacterium(AY858544)
J337(GQ451317)
uncultured Clostridiales bacterium(AM...
J82(GQ451218)
uncultured bacterium(EU460100)
uncultured bacterium(DQ115987)
uncultured bacterium(GQ175476)
J293(GQ451290)
uncultured bacterium(EU772266)
J265(GQ451273)
J36(GQ451193)
J331(GQ451312)
J269(GQ451274)
Pelosinus sp. UFO1(DQ295866)
uncultured bacterium(EU458114)
uncultured bacterium(AF371528)
J19(GQ451187)
uncultured bacterium(AM500844)
J333(GQ451314)
uncultured bacterium(AM275475)
uncultured bacterium(EU843299)
J86(GQ451220)
uncultured bacterium(DQ353915)
Firmincutes
0.02

Figure 3. Phylogenetic tree based on 16S rRNA gene sequences from the *Firmicutes* phyla for *Rhinopithecus bieti*(The scale bar represents 2% sequence divergence).

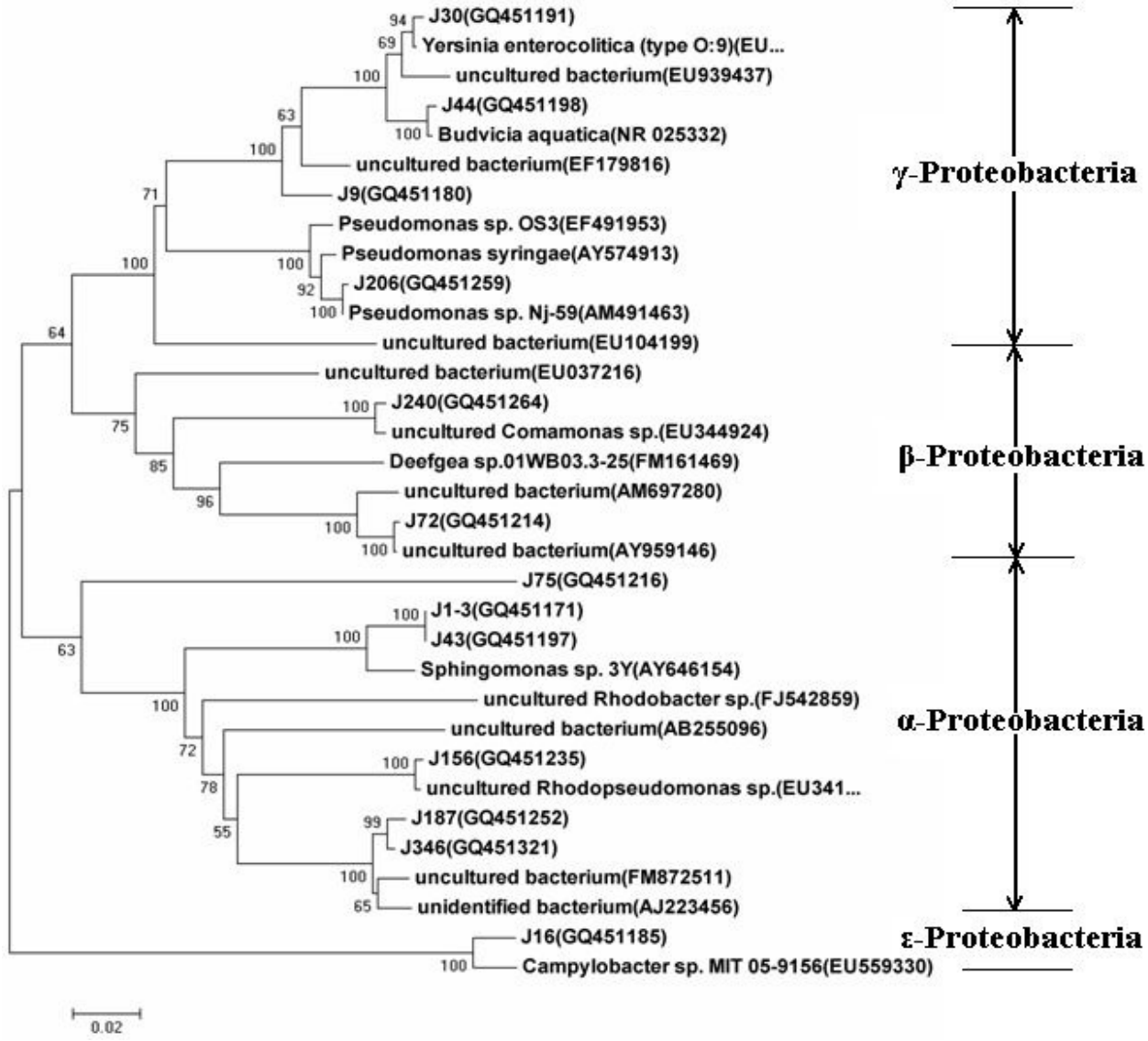

Figure 4. Phylogenetic tree based on 16S rRNA gene sequences from the *Proteobacteria* phyla for *Rhinopithecus bieti* (The scale bar represents 2% sequence divergence).

Budvicia aquatica. Therein, *Yersinia enterocolitica* was the prominent pathogenic bacterium in mammal, and caused enterocolitis and pestilence. J206 was clustered with the three species *Pseudomonas sp. Nj-59*, *Pseudomonas syringae* and *Pseudomonas sp.OS3.* As a result of the close evolutionary distance, it was predicted that J206 belonged to a sub-germline of *Pseudomonas.* J16 had 96% similarity to *Campylobacter* sp. that was isolated from the feces of artificial feeding chimpanzees, and the phylogenetic tree showed that they had a genetic relationship. J240 had 99% similarity with *Comanonas* that was isolated from the intestine of *Hepialus gonggaensis* larvae, and had a bootstrap value of 100% in the phylogenetic tree. J72 had 99% similarity to uncultured bacteria isolated from human vaginal epithelium. In the phylogenetic tree, it clustered with the aerobic bacteria that were isolated from river water in limestone areas, which showed that they were highly related in evolutionary terms.

α-*Proteobacteria* was classified separately as one cluster, which included six clone sequences. J1-3 had 96% similarity with *Sphingomonas* sp. that was isolated from soil polluted with diesel oil. By comparison, J43 shared 99% similarity with unidentified bacteria in human feces, but in the phylogenetic tree, it clustered with *Sphingomonas*. Therefore, in terms of evolution, it had a close genetic relationship with J1-3.

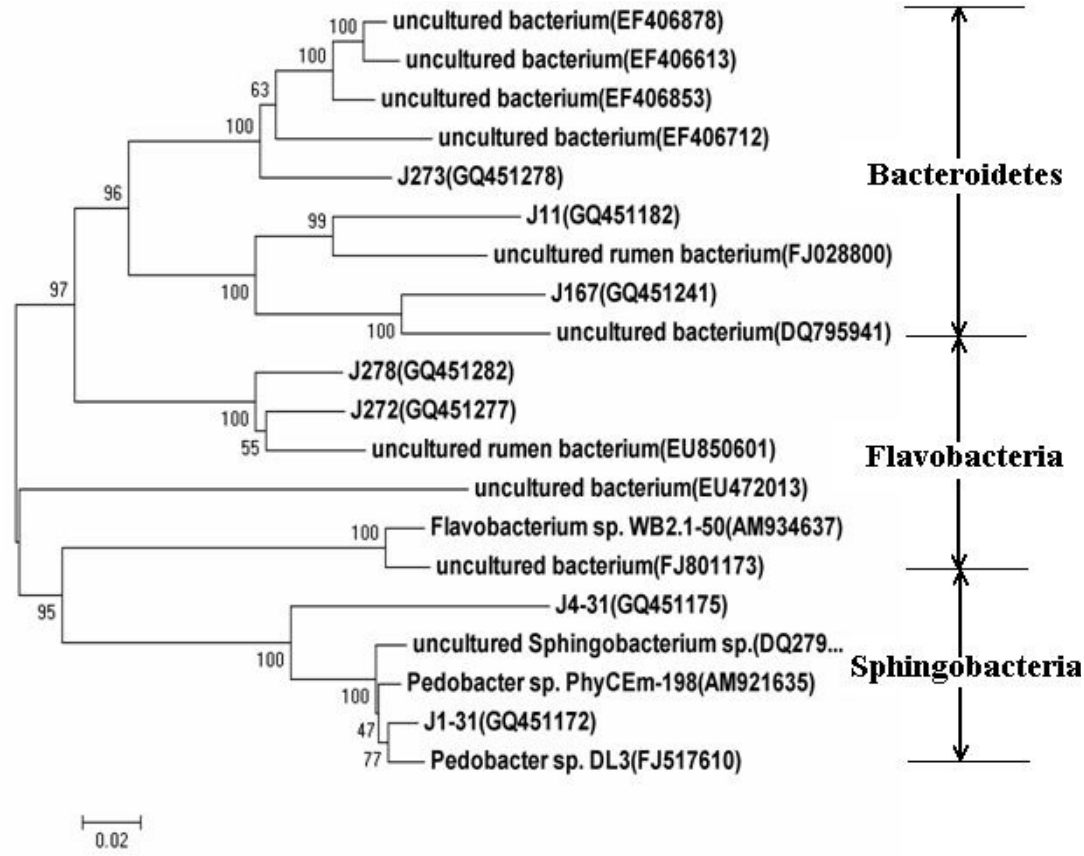

Figure 5. Phylogenetic tree based on 16S rRNA gene sequences from the *Bacteroidetes* phyla for *Rhinopithecus bieti* (The scale bar represents 2% sequence divergence).

Bacteroid, *Flavobacterium* and *Sphingobacterium* classes. By comparison and phylogenetic tree construction, the clone sequences from the intestine of *R. bieti* were classified into three clusters (Figure 5). The three sequences (J273, J11, J167) of the *Bacteroides* class were all uncultured bacteria; two sequences (J272 and J278) belonged to the *Flavobacterium* class; and J4-31 and J1-31 were classified as *Pedobacter* and *Sphingobacterium*, respectively. J273 had 92% similarity with the uncultured bacteria from the mouse colon and they may be relevance to some extent because of their close evolutionary distance. J11 shared a sequence similarity of only 89% with the uncultured rumen bacteria isolated from the rumen of Taiwan water buffalo, but with a 100% bootstrap value, they had a close relationship. As the phenomenon appeared on J11, the J167 though had low similarity to the uncultured bacteria isolated from human feces, they had a close genetic relationship with each other. The research on the bacteria of human feces, which closed genetic relationship to J167, found that *Bacteroidetes* took a relatively lower proportion in the fat men than in the thin men, and *Bacteroidetes* bacteria probably had some link with human obesity (Ley et al., 2006). J272 and J278 had 94 and 93% similarity, respectively, with the uncultured bacteria isolated from rumen fluid of water buffalo, and additionally the phylogenetic tree showed that they had some relativity. Both of them had evolutionary similarity to *Flavobacterium* sp. from river water and uncultured bacteria from marsh water. J4-31 had 89% similarity to *Pedobacter sp. DL3* isolated from south-polar soil. They kept close distance in the phylogenetic tree. J1-31 clustered with *Pedobacter* sp. in the phylogenetic tree, and had 97% similarity to *Pedobacter* sp. isolated from the rhizosphere of *Lolium perenne*, as shown by BLAST analysis.

Phylogenetic analysis of *Fibrobacteres, Spirochaetes and Actinobacteria*

The phylogentic tree was classified into three parts (Figure 6): *Spirochaetes, Fibrobacteres* and *Actinobacteria*, with a total of 30 sequences (20 OTUs). There were seven OTUs (10.9%) classified as *Spirochaetes*. J12 and J215 had 93 and 94% similarity, respectively, to the uncultured bacteria isolated from the rumen fluid of

Taiwan water buffalo, and J270 and others had 92% similarity. J50 had 98% similarity to the uncultured bacteria isolated from excrement of *Nycticebus pygmaeus*, with a 100% boorstrap value. The phylogenetic tree

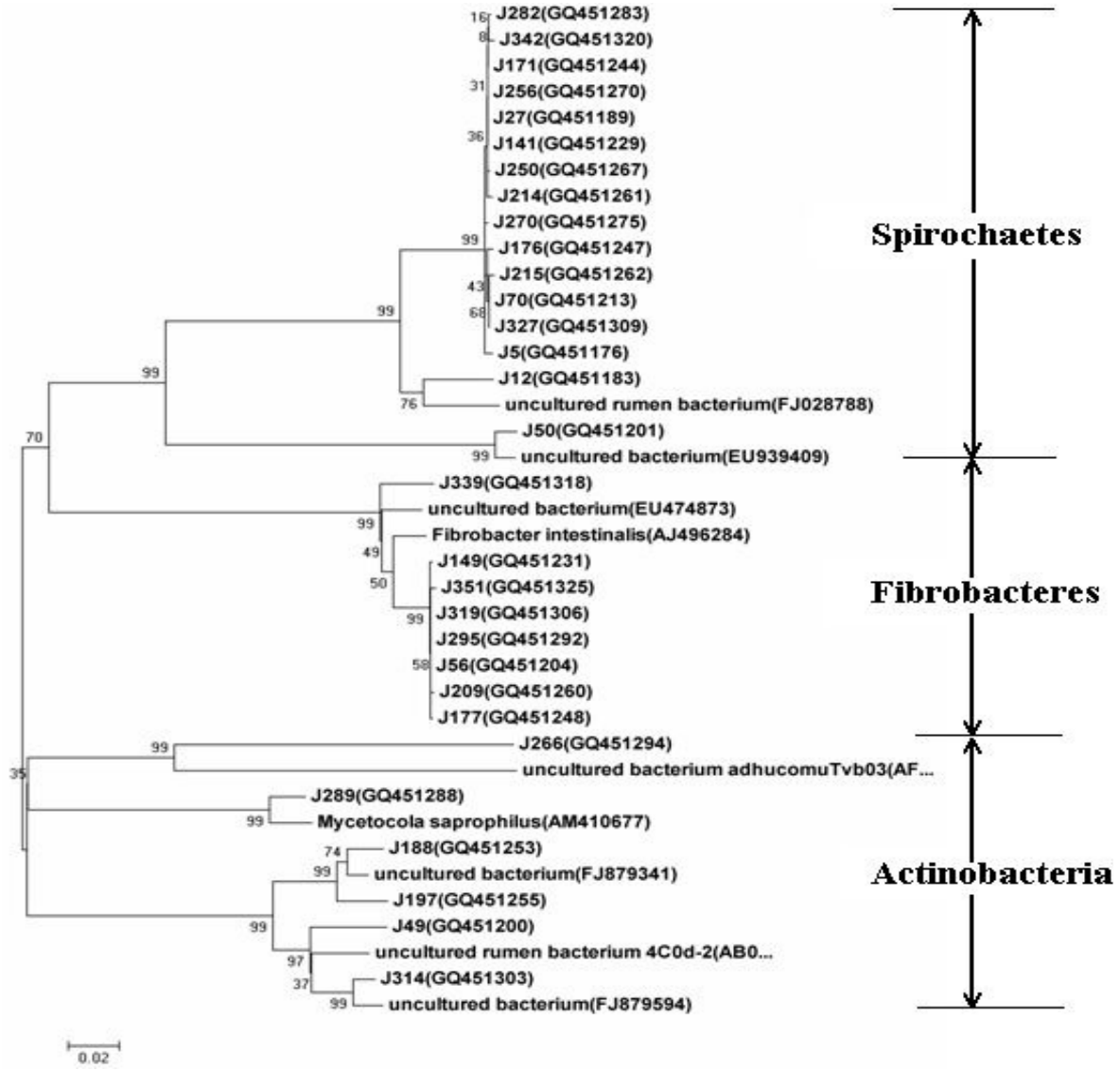

Figure 6. Phylogenetic tree based on 16S rRNA gene sequences from the *Spirochaetes*, Fibrobacteres and *Actinobacteria* phyla for *Rhinopithecus bieti* (The scale bar represents 2% sequence divergence).

showed that these sequences clustered a single branch, which represented their evolutionary relationship.

The second part was classified as *Fibrobacteres* and included eight sequences (7 OTUs), which accounted for 5.13% of the total sequences. The seven sequences J295, J351, J56, J319, J209, 177 and J149 had 96% similarity with *Fibrobacter intestinalis*. J339 had 95% similarity with uncultured bacteria that were isolated from red river hog feces. The phylogenetic tree showed that the eight sequences had a close evolutionary genetic relationship.

The third branch, including five OTUs (0.64%), was classified as *Actinobacteria* (high G+C Gram-positive bacteria). J289 had 97% similarity with *Mycetocola saprophilus*, and the phylogenetic tree demonstrated that they were related in evolution. BLAST showed that J266 shared a sequence similarity of only 89% with *Treponema pectinovorum* (*Treponemas*). However, the phylogenetic tree demonstrated that it had a close genetic relationship with the uncultured bacterium adhucomuTvb03 (AF228819) isolated from human mucous membrane. The four sequences J188, J197, J49 and J314 only matched with uncultured bacteria, and clustered a new branch. This differed from *Mycetocola* which may be a new genus.

Phylogenetic analysis of unidentified *bacteria*

Among the 156 clone sequences, there were 72 sequences (64 OTUs) (46.15%) that did not match with sequences in GenBank, and belonged to uncultured and unclassified bacteria. Independently, they formed a large branch with an extremely high bootstrap value (Figure 1). They possibly represent a novel sub-class or an even higher class. It is predicted that they are special flora that exist among the intestinal bacteria of *R. bieti*. Therefore, future research and development are directed to the under-developed microbial resources. In the phylogenetic tree, they were classified roughly into four clusters, but their taxonomical position could not be defined specifically.

DISCUSSION

Molecular scatology

The *R. bieti* is a rare and valuable endangered species. Therefore, it is impossible to collect samples by conventional methods that involve destructive or invasive sampling. This problem, however, might be solved to a large extent by means of fecal analysis based on molecular scatology in recent years. With the combination of traditional fecal analysis and molecular technology, biologists could make further studies on free-ranging endangered species without disturbing or even observing them (Wei et al, 2001). Fecal samples can be collected easily, and they contain genetic material that can provide much useful information about the animals, which can be used in studies involving micro-genetics, population ecology and behavioral ecology.

Fecal samples of *R. bieti* used in the experiment were soaked in 100% ethanol (Li et al., 2006), transported to the laboratory in an icebox, and stored at –70°C. Although this ethanol preservation possible had little changes in the microbiota of *R. bieti,* it reflects the fecal bacterial diversity of *R. bieti* to a large extent by the use of fecal analysis based on molecular scatology.

Analysis of bacterial diversity

The total DNA extracted from the feces of *R. bieti* was used to construct a 16S rRNA gene library. After homological comparison with the sequences in GenBank, it was found that the intestine of *R. bieti*s had a large number of bacteria that may be involve in the digestion of cellulose, pectin or lignin, such as *Fibrobacter*, *Treponema*, *Pseudomonas*, *Bacillales*, *Clostridium* and *Ruminococcus*. Such as *Fibrobacter intestinalis* can degrade cellulose and hemicelluloses efficiently and specifically (Béra-Maillet et al., 2004), and *Treponema pectinovorum* is able to degrade the cell-wall pectin. Some researchers have found *Bacillus licheniformis* and *Bacillus subtilis*, which play an especially important role in degrading cellulose (Pang, 2004; Hu et al., 2008; Qu et al., 2008). The endocellulase that is generated by *Ruminococcus* and *Clostridium* genera probably is related closely to fiber degradation (Wang, 2008; Zhang et al., 2008; Si and Jiang, 2003). Therefore, the bacterial community that degrade fiber and lignin efficiently and specifically is linked closely with the process by which *R. bieti* digests crude fiber food, such as Chinese usnea, and then transforms it into glycosides or bacterial protein for absorption and utilization.

In addition, *Pedobacter* sp. which generates phytase was discovered (Shao, 2008). This showed that the intestinal bacteria of *R. bieti* can decompose phytic acid and absorb the phosphorus element to promote growth. We also found bacteria such as *Yersinia, Budvicia and Campylobacter*, which were all pathogenic bacteria for humans and animals. *Yersinia* may lead to diarrhea or acute gastroenteritis (Gao et al., 1985). Campylobacter mainly caused chordapsus and food poisoning, as well as adjuvant arthritis, hepatitis and so on. It was unknown whether *R. bieti* suffers from these diseases. The *Sphingomonas* in feces efficiently degraded macromolecular organic pollutants, such as phenanthrene, which had carcinogenic activity in animals and induced an allergic response in the skin. This may help monkeys degrade polluted or poisoned food. J286 had 96% similarity with uncultured bacteria from soil samples polluted by carbon tetrachloride. Strangely, the intestinal flora of *R. bieti* included the anaerobic photoautotrophs, *Rhodopseudomonas* and *Rhodobacter*. However, their functions in the intestine of monkeys were unknown. These probably resulted from the process in which monkeys eat the Chinese usnea and incompletely digested it, which just were the “passers-by” of intestine attached to the food.

Comparison of microbial diversity in feces of animals with different feeding habits

As with any PCR-based method, clone libraries are subject to biases (Von Wintzingerode et al., 1997). With this in mind, the *R. bieti* clone library was compared with gastrointestinal clone libraries from animals using diverse digestive strategies. Table 1 shows the intestinal bacterial diversity of *R. bieti*, Gorillas, Gayals, Normal humans, Vegetarian women, Yaks, and Manchurian tigers. *Actinomycetes, Bacteroidetes* and *Firmicutes* existed in *R. bieti*, Gorillas, and Humans, but the *Firmicutes* in the intestine of *R. bieti* were much less in number than in Humans and Gorillas, rather than the dominant flora. Although *Proteobacteria* were abundant in the intestine of *R. bieti*, they were not present in Gorillas, and only a few were found in the Human intestine. This demonstrated that there was a resemblance between the intestinal flora of *R. bieti*, Humans and Gorillas, but the different feeding habits leaded to obvious discrepancies in the types and numbers of intestinal bacteria in different primates. In

addition, the stomach of *R. bieti* was similar to the rumen of ruminants, therefore, the intestinal flora of *R. bieti* was very similar to that of Gayals and Yaks, but the numbers were significantly different. For instance, *Bacteroidetes,*

Table 2. Comparison of gastrointestinal clone libraries generated from animals with different feeding habits.

Bacterial phylum	% of phylogenetic lineage[a]						
	R. bieti	**Gorilla[b]**	**Gayal[c]**	**Human[d]**	**Vegetarian woman[e]**	**Yak[f]**	**Manchurian tiger[g]**
Actinobacteria	0.64	5.3		0.2	0.5		
Bacteriodetes	4.49	1.1	1.4	47.7	6.0	33.3	
Fibrobacter	5.13					3.3	
Firmicutes	21.15	71.0	57.1	50.8	90.2	58.3	16.3
Fusobacteria				0.08			
Lentisphaerae		3.2					
Planctomycetes		1.1					
Proteobacteria	8.97			0.6	3.3		
Spirochetes	10.9	1.1	0.7			5.1	
Verrucomicrobia		17.2		0.6			
Unclassified	46.15		40.8	0.02			83.7

a: Values are proportions of phylogenetic lineages reported for each clone library.
b: Frey et al. (93 clones from 16 individuals).
c: Deng et al. (147 clones from 6 individuals).
d: Eckburg et al. Data represent the means of the results for three individuals.(11831 clones from 3 individuals).
e: Hayashi et al.（183 clones in total).
f: An et al. (194 clones in total).
g: Tu et al. (15 clones from 2 individuals).

Firmicutes and *Spirochaetes* present in the intestine of Gayals, Yaks and *R. bieti*; however, in the intestine of Gayals and Yaks, *Firmicutes* were the dominant flora. In addition, the *Firmicutes* made up a large proportion of the bacteria in the intestines of *R. bieti*, Gorillas, Gayals, Normal humans, Vegetarian women, Yaks, and Manchurian tigers. This showed that the bacteria of the *Firmicutes* were probably the original flora, which had developed with mammal phylogeny. Whereas, there were a large number of unidentified bacteria in the intestine of *R. bieti* and Gayals. Similarly, there were a large number of unidentified bacteria among the bacterial flora of the Manchurian tiger. These results reveal that the animal intestine bacterial types and numbers differ with the feeding habits of the animals (Table 2).

Conclusion

Based on 16S rRNA gene sequences, we analyzed the composition and distribution of fecal bacteria in *R. bieti*. The bacteria were classified as the following six phyla: *Firmicutes, Proteobacteria, Bacteroidetes, Fibrobacteres, Spirochaetes and Actinobacteria*. The 156 different kinds of 16S rRNA gene sequences were classified into 17 genera and 122 uncultured and unidentified bacteria. This demonstrated the diversity of intestinal bacteria in *R. bieti* and made up for the defects and deficiencies of the traditional culture methods in analyzing microflora. However, 16S rRNA gene sequence analysis is not sufficient to guarantee the clarification of the microbiological environment, because variation always occurs in the extraction of DNA, PCR and cloning selection. Therefore, the culture-independent approach should be combined with direct morphological observation and *in-situ* hybridization to improve the efficiency of research.

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Full Length Research Paper

Quality assessment of plantain (*Musa paradisiaca* L.) as affected by different ripening methods

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There are increasing reports of food poisoning due to preservatives used for the processing of certain food items, especially in developing countries of Africa. Also, very scanty information is available on the effect of these preservatives on the nutritional status of the food being processed and preserved. This experiment therefore reports on the quality assessment of plantain (*Musa paradisiaca* L.) as affected by different ripening agents used to accelerate the period of plantain ripening. The experiment consisted of 4 ripening agents, namely: calcium carbide, *Irvingia gabonensis* fruits, *Newbouldia laevis* leaves and control, where no ripening agents were applied to the blossoms of plantain. The unripe and ripened blossoms of plantain were analyzed for their physicochemical properties using standard methods. Ripened plantains without any ripening accelerator had significantly ($p < 0.05$) higher values of crude protein (3.51%), crude fat (0.33%), total ash (2.55%), crude fiber (0.42%) and reducing sugar (10.42%) when compared with other treatments. Least values of this proximate composition (crude protein, crude fat, total ash, crude fiber and reducing sugar) with no significant difference were obtained when calcium carbide (1.31, 0.04, 1.28, 0.04 and 7.07%) and *Irvingia gabonensis* (1.53, 0.06, 1.04, 0.03 and 9.17%) were applied, respectively. It was concluded that since these ripening agents have adverse affects on the nutritional status of plantains, an effective food safety program and control measures need be put in place to monitor various methods of plantain ripening with a view to ultimately safeguarding public health.

Key words: Calcium carbide, food poisoning, *Irvingia gabonensis, Newbouldia laevis*, plantain blossoms.

INTRODUCTION

Food contamination through poisons is on the increase in Nigeria (Ali, 2009). The rate is becoming alarming with the way people died after the consumption of certain food items. Awofadeji (2008) reported the death of two people after they had eaten cooked beans in Calabar, Nigeria. Adeleke (2009) also reported on the use of certain lethal preservatives for the processing of yam flour which eventually caused food poisoning among three families in Kano, Nigeria. 'Amala', a local diet in Nigeria is prepared from the poisoned yam flour. Adeleke (2009) reported further that the affected people however, recovered after treating them of diarrhea, vomiting, abdominal pain and convulsion. Complaints about the ripening methods used on mature plantain blossoms before they ripened are on the increase. Most often, in West Africa, mature plantain bunches are harvested just before they begin to ripe and thereafter sold to market women.

Blossoms of plantain are consumed as a vegetable by most people in Nigeria raw, boiled, roasted or fried with rice/beans. The unripe but mature blossoms of plantain are sometimes processed to flour for other diets. The over-ripened plantains are even processed into a local wine called 'agadangidi'. Asiedu (1980) reported that the blossoms of plantain are consumed at five different stages of ripeness.

The National Agency for Food, Drugs and Administrative Control (NAFDAC) in Nigeria that is charged with the responsibility of enforcing all laws, guidelines, policies and compliance that sub-standard or adulterated food and drugs are not found in the country concentrates mostly on 'canned' items. People take advantage of this inadequacy to process and preserve 'uncanned' food items, including plantain blossoms without any regard to

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Table 1. Physicochemical properties of plantain blossoms.

Property (%)	Treatment				
	UP	RP1	RP2	RP3	RP4
Crude protein	4.82 ± 0.02a	1.34 ± 0.02b	1.31 ± 0.00b	1.53 ± 0.22b	3.51±0.44a
Moisture content	59.25 ± 0.75b	61.25 ± 0.75b	64.99± 0.55a	64.89±0.11a	64.35±0.30a
Fat	0.62 ± 0.02a	0.55 ± 0.02a	0.04 ± 0.00c	0.06 ± 0.00c	0.33 ± 0.01b
Total ash	2.79 ± 0.01a	2.47 ± 0.25a	1.28 ± 0.02b	1.04 ± 0.02b	2.55 ± 0.20a
Crude fiber	0.57 ± 0.02a	0.38 ± 0.03a	0.04 ± 0.00b	0.03 ± 0.00b	0.42 ± 0.02a
Carbohydrate	31.78 ± 0.59b	33.99 ± 1.22a	32.35±0.01ab	32.45±0.10ab	28.88±0.43b
Dry matter	40.75 ± 0.75a	38.75 ± 1.25b	34.51±0.49c	35.11±0.11c	35.15±0.20c

Values within a row followed by different letter(s) are significantly different according to new Duncan Multiple Range Test at $p < 0.05$.
UP = Unripe plantain blossoms, RP1 = ripened plantain blossoms using *N. laevis* leaves as ripening agent, RP2 = ripened plantain blossoms using calcium carbide as ripening agent, RP3 = ripened plantain blossoms using *I. gabonensis* fruit as ripening agent and RP4 = ripened plantain blossoms with zero ripening agent as control.

methods being used. *Newbouldia laevis* leaves and *Irvingia gabonensis* fruits are now being used as ripening agents to accelerate the ripening period by the local farmers. Previous works on these plants had always been on their medicinal importance. Ogunlana and Ogunlana (2008) reported the antioxidant property in *N. laevis* stem bark. Also, the fresh fruit of *I. gabonensis* is found useful in the treatment of Type II diabetics and in reducing obesity (Judith et al., 2005). Ndjouenekeu et al. (1996) worked on the usefulness of the kernels of *I. gabonensis* as a condiment and food thickening property in preparing draw soup locally called 'ogbono'. The study location, Ile- Ife, is a semi-urban city in Osun State and had earlier been mapped by Enwezor et al. (1989) as one of the restricted areas where plantain grows well in Nigeria. This study, therefore attempts to assess the quality of plantain (*Musa paradisiaca* L.) as affected by different ripening methods commonly used and give recommendation on the best method that could be adopted and promoted to the market as an ideal ripening method for blossoms of plantain.

MATERIALS AND METHODS

This study was conducted in the Institute of Ecology and Environmental Studies, Food Analytical Laboratory and Central Services Laboratory of the Obafemi Awolowo University, Ile-Ife, Nigeria in 2009. Preliminary studies were carried out to establish the ripening methods commonly used in the study area prior to the commencement of the main work, since no studies have previously been reported on it. The preliminary studies identified three commonest plantain ripening agents used to accelerate the ripening period. These are: *N. laevis* (Seem) leaves, *I. gabonensis* (Aubry-Lecomte, Baill.) fruits and calcium carbide.

Freshly harvested and mature, but unripe one plantain bunch that contained 40 blossoms was procured from a fruit orchards private farmer at the Obafemi Awolowo University, Ile-Ife, Nigeria. With the help of a clean kitchen knife, the plantain blossoms were carefully separated from the bunch.

In this study, the three identified ripening agents and the control (zero ripening agent) made up the four treatments were used. Each treatment was replicated three times to give a total of 12 replicates. Each replicate contained three blossoms of plantain of relatively uniform weight (700.00 g). Also, relatively uniform weights (0.50 g) of the ripening agents were used to either wrap the blossoms (*N. laevis* leaves) or drop with the blossoms [*I. gabonensis* fruits and calcium carbide (wrapped with transparent nylon)] and each replicate was put inside a bag and tied up. All the replicates were stored in the same room to ripe.

Daily room temperature and time taken for the plantain blossoms to ripe were recorded. The physicochemical properties of unripe and ripened plantain blossoms were determined according to the standard methods of Association of Official Analytical Chemists (AOAC, 1990). The remaining blossom samples were visually observed for the presence of fungal colonies and deterioration rates were monitored till 4 days after the blossoms have fully ripened.

RESULTS AND DISCUSSION

Table 1 presents a summary of the physicochemical properties of unripe and ripened plantain blossoms using different ripening agents. The crude protein of unripe plantain was 4.82% while the protein content of ripened plantain ranged from 1.31 to 3.51%. There was no significant difference in the crude protein of unripe and ripened blossoms of plantain when no ripening agent (RP4) was used. However, significant ($p < 0.05$) reduction in protein contents were recorded when ripening agents were used. In fact, less than 50% of the protein content was retained in plantain when any of the ripening agents was used to accelerate the ripening period. The ripening agents might have contributed positively to the loss of nitrogen.

The moisture contents of the ripened blossoms varied from 61.25 to 64.99% which were higher than the unripe (59.25%) blossoms. The ripened plantain due to the *N. laevis* leaves (RP1) had the least moisture content of 61.25%. This may be attributed to the fact that as the leaves were decaying, they absorbed moisture from the plantains. Crude fat of the ripened plantains varied from 0.04 to 0.55%. These were significantly ($p < 0.05$) lower than the crude fat of unripe plantain. Over 50% of the fat was retained in the ripened plantain when no ripening agent

Table2. Vitamin C, sugar and pH values of plantain blossoms.

Treatment	Property				
	UP	RP1	RP2	RP3	RP4
pH	5.87 ± 0.03a	4.94 ± 0.22a	5.37 ± 0.20a	5.28 ± 0.24a	5.05 ± 0.15a
Brix (%)	0.00 ± 0.00d	20.00 ± 0.00a	20.00 ± 0.00a	18.00 ± 2.00b	15.00 ± 0.15c
Reducing sugar (%)	0.00 ± 0.00c	10.30 ± 0.20a	7.07 ± 0.23b	9.17 ± 0.25ab	10.42 ± 0.25a
Vitamin C (mg/100 g)	4.24 ± 0.20c	9.09 ± 0.15a	8.48 ± 0.17ab	7.27 ± 0.20ab	6.06 ± 0.10b

Values within a row followed by different letter(s) are significantly different according to new Duncan Multiple Range Test at $p < 0.05$.
UP = Unripe plantain blossoms, RP1 = ripened plantain blossoms using *N. laevis* leaves as ripening, RP2 = ripened plantain blossoms using calcium carbide as ripening agent, RP3 = ripened plantain blossoms using *I. gabonensis* fruit as ripening agent and RP4 = ripened plantain blossoms with zero ripening agent as control.

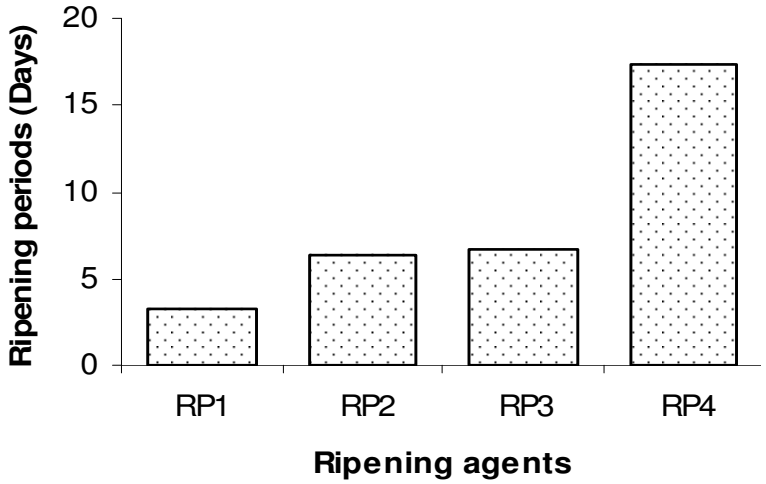

Figure 1. Ripening periods of blossoms of plantain as affected by different ripening agents. RP1 = Ripened plantain blossoms using *N. laevis* leaves as ripening agent; RP2 = ripened plantain blossoms using calcium carbide as ripening agent; RP3 = ripened plantain blossoms using *I. gabonensis* fruit as ripening agent; RP4 = ripened plantain blossoms with zero ripening agent as control.

(0.33%) was used while with carbide (RP2) (0.04%) and *I. gabonensis* fruits (RP3) (0.06%) the fat contents were exceedingly low.

Total ash of the ripened plantains ranged from 1.04 to 2.55 %. The total ash of RP4 and RP1 were not significantly different, while in RP2 and RP3, the total ash significantly ($p < 0.05$) reduced. The crude fiber, which is the bulk of roughages in food was moderate in all plantains, but very low with RP2 and RP3 which may be due to loss of nutritional values as a result of these ripening agents.

The carbohydrate contents in the ripened plantains ranged from 28.88 to 33.99% while the unripe plantain had 31.78%. Only RP1 showed significant higher value (33.99%) when compared with control (RP4) having 28.88% carbohydrate. The dry matter content of the ripened plantains followed this pattern also.

The pH values of the ripened plantains ranged from 4.94 to 5.37 showing slight acidity as shown in Table 2. There was no significant difference in the pH values of the ripened plantains by the different ripening agents, though the pH with RP1 had the least while with RP2 had the highest value. The calcium ions in carbide may have enhanced this alkalinity condition. Total (brix) sugar and available (reducing) sugar of the unripe plantains was zero. This is normal; since it is the breaking down of carbohydrates which occur during ripening gives rise to sugar. After full ripening of the plantains, RP4 gave the highest value of available sugar (10.42%) while RP2 gave the significantly ($p < 0.05$) lowest (7.07%) value. The vitamin C in ripened plantains with RP1 was the highest, which however, was not significant when compared with RP2 and RP3 but significantly ($p < 0.05$) higher than in RP4.

Figure 1 gives the period it takes each of the treatments to ripe after the application of the ripening agents. Figure 2 give the plantains' conditions before and 4 days after ripening. RP1 was the first to ripe while RP4 ripened

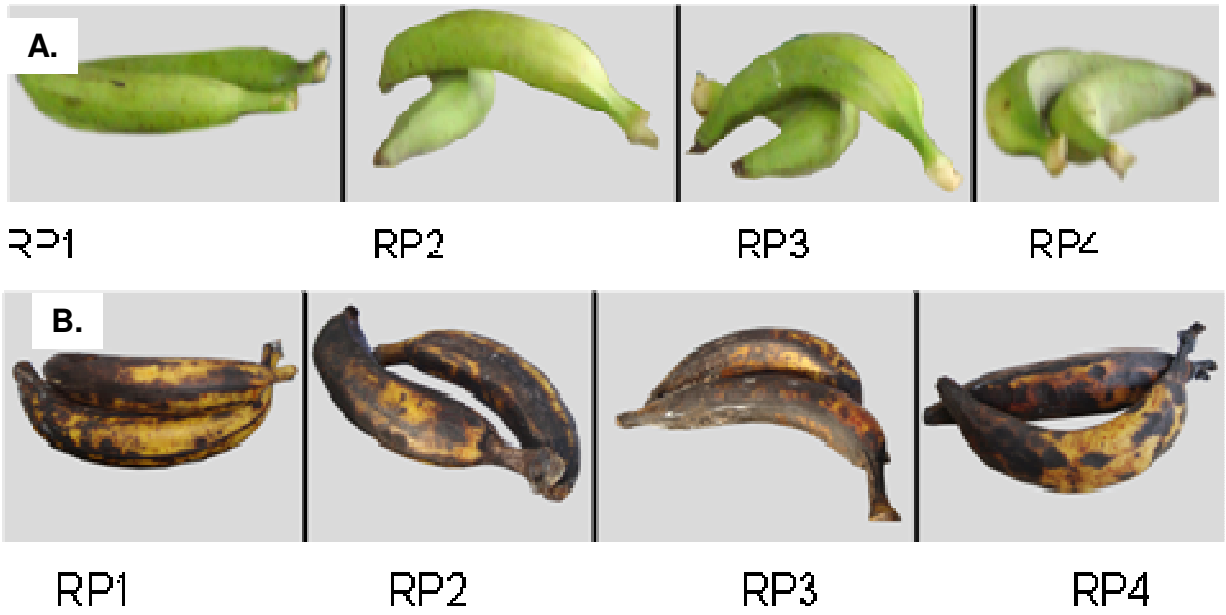

Figure 2. A. Unripe plantain blossoms. **B.** Plantain blossoms 4 days after ripening using different ripening agents. RP1 = Ripened plantain blossoms using *N. laevis* leaves as ripening agent; RP2 = ripened plantain blossoms using calcium carbide as ripening agent; RP3 = ripened plantain blossoms using *I. gabonensis* fruit as ripening agent; RP4 = ripened plantain blossoms with zero ripening agent as control.

last. This confirms the effectiveness of the ripening agents over zero application. However, four days after the blossoms of plantain have fully ripened, the visual observation on them confirmed the presence of fungal colonies in the order: RP2 > RP3 > RP1, whereas, RP4 had no fungi infestation.

Conclusion and recommendation

This study has shown the effect of ripening agents on the quality of the ripened blossoms of plantain. It has provided empirical data on the physicochemical properties of unripe and ripened plantains. There is evidence of nutritional quality reduction when ripening agents are used to accelerate the period of ripening of the blossoms of plantain. Low level of ignorance on the part of the farmers about what these ripening agents can do is capable of preventing the plantain from making its full nutritional values available to the consumers. An effective food safety programme must be put in place by the government(s) to seriously monitor various methods of plantain ripening so as to guide against anything that would have negative impact on human health.

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Full Length Research Paper

LeERF1 improves tolerance to drought stress in tomato (*Lycopersicon esculentum*) and activates downstream stress-responsive genes

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Ethylene responsive factors (ERFs) are important transcriptional regulators involved in plant responses to abiotic and biotic stresses. In this study, the function of the *LeERF1* gene in sense- and antisense-*LeERF1* transgenic tomato plants was analyzed. The results demonstrated that overexpression of *LeERF1* in tomato plants enhanced tolerance to drought stress. The *LeERF1*-overexpressing transgenic plants maintained higher relative water content (RWC), free proline and soluble sugar levels, and showed lower malondialdehyde (MDA) level and electrolyte leakage under drought stress, compared with wild-type and antisense-*LeERF1* tomato plants. Furthermore, overexpression of *LeERF1* in tomato plants activated the expression of stress-related genes, including *P5CS*, *LEA*, *Itpg2* and *tdi-65*. These results suggested that *LeERF1* played a positive role in tolerance to drought stress.

Key words: *LeERF1*, drought tolerance, transcription factor, transgenic tomato.

INTRODUCTION

Ethylene is a gaseous phytohormone that plays an important role in the regulation of growth processes, including seed germination, fruit ripening, and organ senescence and abscission, which are involved in stress and pathogen attack (Johnson and Ecker, 1998; Bleecker and Kende, 2000). Therefore, it is crucial to study the function of ethylene to understand the stress response. The ethylene responsive element binding proteins, first discovered in tobacco as binding proteins, which contain a highly conserved DNA binding domain known as the ethylene responsive factors (ERF) domain (Ohme-Takagi and Shinshi, 1995). The ERF domain is composed of an α-helix and a β-sheet that interacts with the target DNA (Allen et al., 1998). The ERF protein binds to the *cis*-acting element AGCCGCC (GCC box) as the core sequence essential for defense-related genes (Hao et al., 1998).

A large number of ERF proteins were identified from various plants including *Arabidopsis*, tobacco, wheat, rice and hot pepper. For example, overexpression of *AtERF14* in *Arabidopsis* has largely enhanced defense gene expression and regulated the expression of other ERF genes (Onate-Sanchez et al., 2007). *GbERF2* transgenic tobacco plants accumulated higher levels of pathogenesis-related gene transcripts and enhanced resistance to fungal infection (Zuo et al., 2007). Over-expression of *TaERF1* in wheat activated stress-related genes and improved pathogen and abiotic stress tolerance in transgenic plants, suggesting that *TaERF1* might be involved in multiple stress signal transduction pathways (Xu et al., 2007). The expressions of four rice ethylene-responsive transcription factors *OsBIERF1-4* enhanced biotic and abiotic stress (Cao et al., 2006).

In tomato, several ERF proteins have been identified in response to biotic and abiotic stresses. For example, the

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Abbreviations: ERFs, Ethylene responsive factors; **RWC,** relative water content; **MDA,** malondialdehyde; **PR,** pathogenesis-related; **RT-PCR,** reverse transcriptase polymerase chain reaction; **FW,** fresh weight; **TM,** turgid mass; **DW,** dry weight; **MDA,** malondialdehyde.

tomato Pti4-6, specially recognized and bound to a DNA sequence that was present in the promoter region of a large number of genes encoding “pathogenesis-related” (PR) proteins (Zhou et al., 1997). Expression of tomato *JERF3* in tobacco activated the expression of oxidative and osmotic stress-related genes, resulting in decreased accumulation of reactive oxygen species (ROS) and enhanced adaptation to drought, freezing and salt stress (Wu et al., 2008). Tomato transcription factor, JERF1, interacted with multiple *cis*-acting elements and activated the expression of stress responsive genes, ultimately enhancing tobacco tolerance and growth under high salinity and low temperature (Wu et al., 2007). Tomato TERF1 can interact with both GCC-box and DRE to enhance the expression of genes involved in biotic and abiotic stress tolerance (Huang et al., 2004). These results indicated that ERF proteins interacted with down-stream partners of various stress-responsive genes, sub-sequently conferred responses against biotic and abiotic stresses. Previously, an ERF protein LeERF1 was isolated from a cDNA library of tomato fruit, which contained a conserved ERF domain of specific binding to the *cis*-acting element GCC box (Yu et al., 2004). We also obtained sense- and antisense-*LeERF1* transgenic tomato plants, in which *LeERF1* positively modulated ethylene triple response on etiolated seedling, plant development, fruit ripening, and softening in tomato (Li et al., 2007).

In the present investigation, we found that sense-*LeERF1* transgenic tomato plants showed much more tolerance to drought stress, compared with wild-type and antisense-*LeERF1* transgenic tomato plants. This paper aims to investigate the physiological changes and expression of downstream stress-related genes under drought stress. The results suggest that *SlERF1* might play a key role in tolerance to drought stress.

MATERIALS AND METHODS

Plant materials and growth conditions

Tomato (*Lycopersicon esculentum* cv Zhongshu No.4) plants were grown in the growth chamber at 25°C under 16/8 h light/dark cycle, with a relative humidity of 70%. Four-week-old tomato seedlings (T_3 generation) were used for the drought stress treatment and quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Wild-type tomato plants and transgenic tomato plants of sense- and antisense-*LeERF1* were designated as WT, LeERF1-sn and LeERF1-as, respectively.

Analysis of drought tolerance in transgenic tomato plants

The T_3 generation of LeERF1-sn, LeERF1-as and WT lines were treated under drought stress condition. For drought stress treatment, the plants were transplanted to pots filled with soil and vermiculite (1:1). Four-leaf stage tomato seedlings were withheld water for 10 days. Leaves of the tomato plants were harvested, frozen in liquid nitrogen and stored at -80°C until required. About 30 seedlings were used for each transgenic line.

Measurement of the relative water content

The relative water content (RWC) of the wild-type and LeERF1 transgenic tomato plants was performed as described by Yamasaki and Dillenburg (1999). Leaves were obtained from the tomato plants and their fresh weight (FW) was measured. To determine turgid mass (TM), the leaves were floated in deionized water for 7 h. Finally, the leaf samples were dried in an oven at 80°C for 48 h to obtain the dry weight (DW). The RWC was then calculated using the following formula:

$$RWC\ (\%) = [(FW\text{-}DW) / (TW\text{-}DW)] \times 100$$

Assay of malondialdehyde (MDA) content and electrolyte leakage

MDA content was estimated by thiobarbituric acid (TBA), as described by Peever and Higgins, (1989). The absorbance was determined at 450, 532 and 600 nm, respectively. The content of MDA was calculated as indicated:

$$C\ (\mu mol\ l^{-1})_{MDA} = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$$

Electrolyte leakage assay of the leaves of the transgenic and wild-type tomato plants was conducted according to the method of Dionisio-Sese and Tobita (1998). The conductivity of the solution was determined by a conductivity meter (Model DDSJ-308A, Shanghai Precision & Scientific Instrument Co., Ltd., China).

Measurement of the proline and soluble sugar contents

Proline was determined as described by Bates et al., (1973). The absorbance was measured at 520 nm and proline content was calculated using the standard curve. The content of soluble sugar was determined at 620 nm according to the method of Morris (1948).

Gene expression analysis

Total RNA was extracted from the four-week-old tomato seedlings following the procedure of guanidium thiocyanate (Gregory et al., 1988). RNA treated with DNase was reverse transcribed using M-MLV reverse transcriptase (Promega, USA) with a poly-T primer, according to the instructions of the manufacturer. Quantitative RT-PCR was performed on an ABI PRISM 7000 sequence detection system (Applied Biosystems) by using SYBR Green PCR Master Mix (Toyobo, Japan). The qRT-PCR amplification conditions were set as follows: initial denaturation of 5 min at 95°C, 40 cycles of at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, with a final extension of 10 min at 72°C. *Actin* was used as a reference gene in each PCR reaction. Each sample was performed with three replicates. The following gene specific primers were used: 5´-TGGGATGATATGGAGAAGATATGG-3´ and 5´-GGCTTCAGT TAGGAGGACAGGA-3´ for *Actin* (U60480); 5´-CGTCCTCTGTT GTCCC-3´and 5´-AGTGAAGGCAATGAAGC-3´ for *tdi-65* (AF 172856); 5´-GTCCCAATCTCCTCCAA-3´ and 5´-CAGGGTAAT CGCATCAG-3´ for *LEA* (Z46654); 5´-TACTGGACC GTTGAGCA-3´ and 5´-GGTGTTGTGGTGGTGTTA-3´ for *ltpg2* (U81996); 5´-CCCACAGCAGCACAA-3´ and 5´-TTCGCAAGGGTATGAAG-3´ for *P5CS* (AY897574).

Statistical analysis

Statistical analyses were carried out via one-way analysis of variance

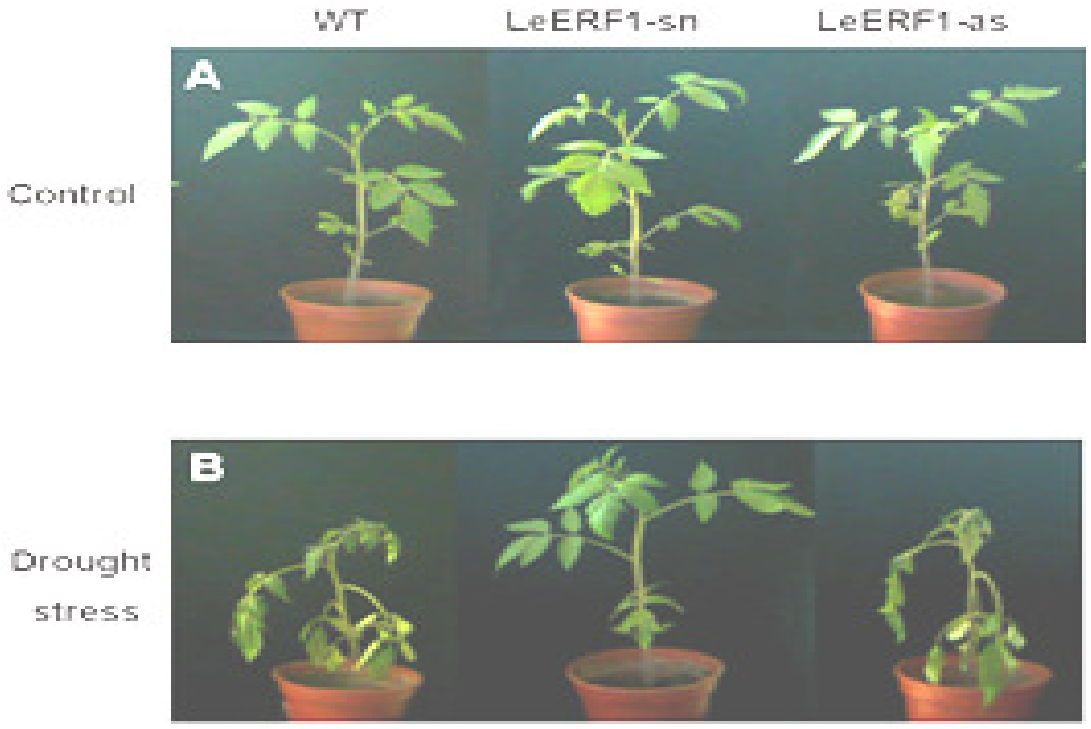

Figure 1. Evaluation of drought tolerance in wild-type (WT), sense-*LeERF1* (LeERF1-sn) and antisense-*LeERF1* (LeERF1-as) tomato plants. Four-week-old tomato seedlings were withheld in water for 10 days. About 30 seedlings were used for each WT and *LeERF1* transgenic tomato plants.

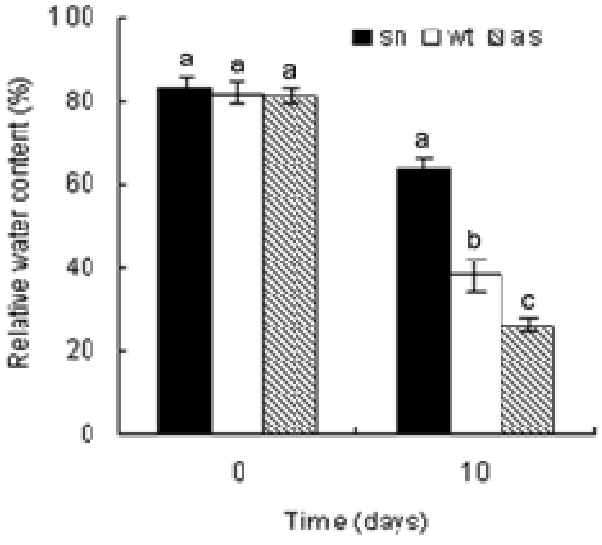

Figure 2. The relative water content of wild-type (WT), sense-*LeERF1* (sn) and antisense-*LeERF1* (as) transgenic tomato plants grown under drought stress for 10 d. The bars represent the mean ± SD of three individual measurements. Columns with different letters indicate significant difference at $P<0.05$ (Duncan test).

and Duncan's multiple range test. The statistical significant was evaluated at the $P<0.05$ level.

RESULTS

Overexpression of *LeERF1* in tomato plants enhanced tolerance to drought stress

To investigate the response of *LeERF1* transgenic plants to drought stress, the four-week-old seedlings were grown under water-deficit stress for 10 d. After drought-stress, the leaves of the WT and LeERF1-as transgenic lines wilted, rolled and exhibited chlorosis, while only a few of the leaves of LeERF1-sn transgenic lines rolled (Figure 1). To further study the capacity of osmotic adjustment in transgenic tomato plants, relative water content was measured under drought stress condition. The results showed that no significant differences exist between WT and transgenic plants under normal condition. However, the RWC of LeERF1-sn plants was reduced to 20%, whereas WT and LeERF1-as plants declined to 43.7 and 54.9% under drought stress, respectively (Figure 2). These results suggested that LeERF1-sn transgenic tomato plants were more tolerant to drought stress, compared with WT and LeERF1-as plants.

Overexpression of *LeERF1* in tomato plants maintained the stability of cell membrane

To study the stability of membrane in transgenic and WT

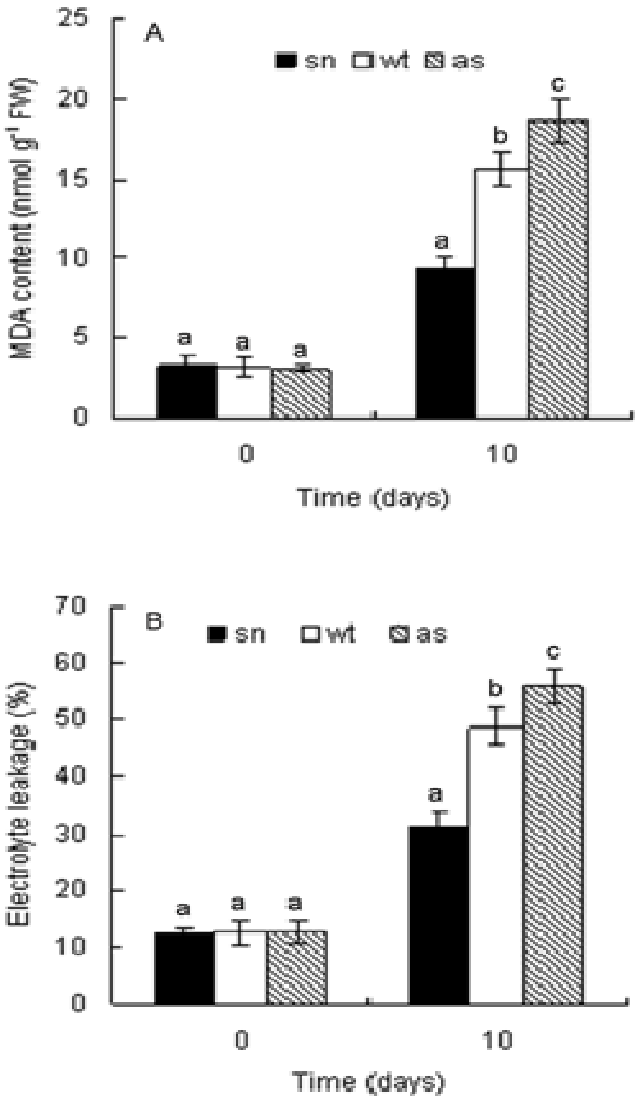

Figure 3. MDA content (A) and electrolyte leakage (B) of wild-type (WT), sense-*LeERF1* (sn) and antisense-*LeERF1* (as) tomato plants under drought stress for 10 d. The results are the mean ± SD of three individual measurements. Columns with different letters indicate significant difference at $P<0.05$ (Duncan test).

plants under drought stress, MDA content and electrolyte leakage as an indication of membrane damage were determined. The results indicated that MDA content and electrolyte leakage increased in tomato plants under drought stress. However MDA content and electrolyte leakage in WT and LeERF1-as plants increased more than those in LeERF1-sn transgenic lines. The amount of MDA in LeERF1-sn transgenic plants increased only 2.7-3.1 folds, compared to non-treated plants under drought stress (Figure 3a). Electrolyte leakage increased 31.3% in LeERF1-sn transgenic plants, thus in WT and LeERF1-as lines increased 48.7 and 55.8% under drought stress, respectively (Figure 3b). The results indicated that the LeERF1-sn transgenic tomato plants were subjected to less damage to the cell membrane under drought stress.

Accumulation of free proline and soluble sugar in sense-*LeERF1* transgenic tomato plants

Proline and soluble sugar are osmolyte in plants and accumulate in response to stress treatment. After being withheld in water for 10 days, the proline content in both *LeERF1* transgenic lines and WT lines increased constantly (Figure 4a), whereas the level of proline in the LeERF1-sn lines was significantly higher than that in WT and LeERF1-as lines. In the LeERF1-sn transgenic tomato plants, the proline content increased 4.6 - 4.7 folds under drought stress, compared with the non-treated lines. However, in the WT and LeERF1-as tomato plants, the proline content was only increased from 3.0 - 3.5 folds and 2.4 - 2.7 folds compared with the non-treated lines, respectively. The levels of soluble sugar in the LeERF1-sn, WT and LeERF1-as lines under drought stress showed, respectively, 6.3 - 7.4 folds, 2.6 - 3.6 folds and 2.8 - 3.7 folds higher than those of the non-treated lines (Figure 4b). There was no significant difference in the levels of free proline and soluble sugar between WT and LeERF1 transgenic lines under normal condition.

Activation of stress-related genes in *LeERF1*-overexpressing transgenic tomato plants

Overexpression of *TERF1* in rice activated the expression of stress-responsive genes and enhanced tolerance to abiotic stress (Gao et al., 2008). To further elucidate the role of *LeERF1* gene involved in drought stress, we analyzed the expression of several stress-related genes, such as *P5CS*, *LEA*, *ltpg2* and *tdi-65* in WT and *LeERF1* transgenic lines. As shown in Figure 5, the expression levels of these genes in LeERF1-sn transgenic lines were higher than those in WT plants under normal condition. Especially, the expression level of *ltpg2* in LeERF1-sn plants was nearly 13 folds compared to WT lines under normal condition. In contrast to WT and LeERF1-as plants, the expression levels of these four genes were significantly activated in LeERF1-sn transgenic tomato plants under drought stress.

DISCUSSION

Majority of studies demonstrated that ERF proteins played an important role in plant abiotic and biotic stress responses and developmental processes (Chakravarthy et al., 2003; Alonso and Stepanova, 2004). Our cloned *LeERF1* gene encoded the conserved ERF domain of specific binding to the *cis*-acting element GCC box (Yu et al., 2004). We have described previously that the ERF transcription activator *LeERF1* positively mediated

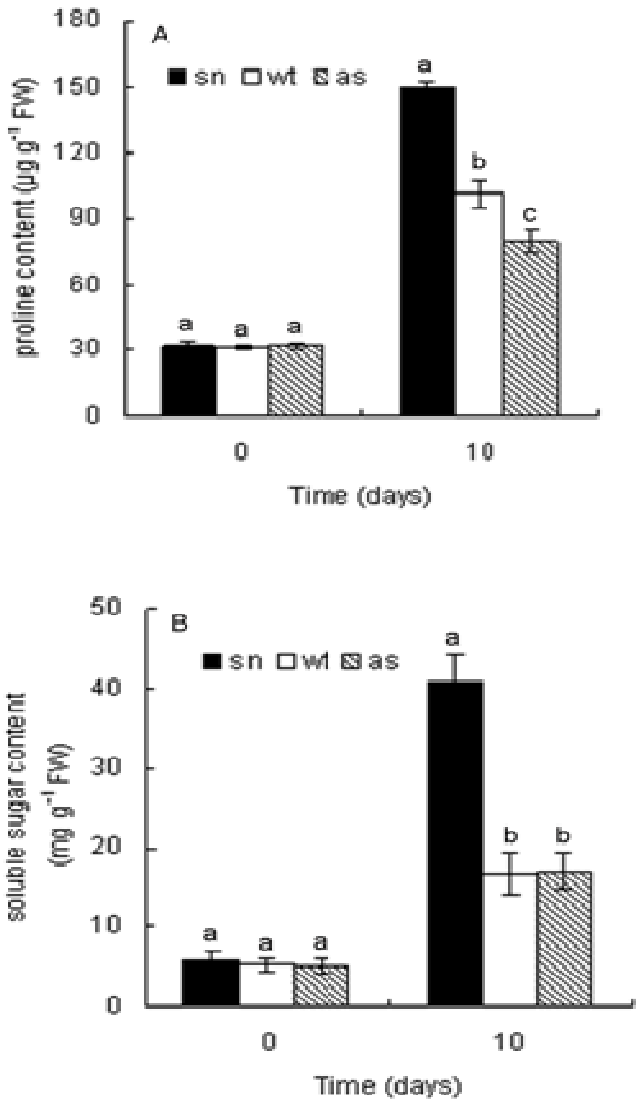

Figure 4. Levels of proline (A) and soluble sugars (B) in wild-type (WT), sense-*LeERF1* (sn) and antisense-*LeERF1* (as) tomato plants under drought stress for 10 d. The results are the mean ± SD of three individual measurements. Columns with different letters indicate significant difference at $P < 0.05$ (Duncan test).

ethylene signals and was a downstream component in the ethylene signaling pathway in tomatoes (Li et al., 2007). In this study, overexpression of *LeERF1* in tomato plants enhanced tolerance to drought stress and up-regulated downstream stress-related genes.

Plants respond to abiotic and biotic stress in their natural environment, affecting various physiological and biochemical changes (Fujita et al., 2006; Knight and Knight, 2001). Our results showed that WT and LeERF1-as tomato plants displayed growth inhibition, chlorosis and rolled under drought stress, whereas the LeERF1-sn transgenic lines grew normally (Figure 1). RWC as a general measure of plant water status was determined.

The results indicated that LeERF1-sn transgenic tomato plants maintained a significantly higher RWC in their leaf tissue, as compared with the WT and LeERF1-as lines (Figure 2). Furthermore, abiotic stresses can cause oxidative damage to cell membrane (Chinnusamy et al., 2005). It has been shown that constitutive expression of *OsDREB1B* in tobacco plants showed significantly lower MDA content than the wild type plants under osmotic stress (Linga and Arjula, 2008). In this study, the levels of MDA and electrolyte leakage in WT and LeERF1-as tomato plants were higher than those in LeERF1-sn transgenic plants (Figure 3). It implied that over-expression of *LeERF1* in tomato plants maintained the stability of cell membrane under drought stress condition.

Under a variety of abiotic stress, plants accumulated osmolytes including proline and soluble sugar (Bonhert and Jensen, 1996). These osmolytes function as osmo-protectants to improve tolerance to stress responses by their own protective mechanisms (Hasegawa et al., 2000; Bhatnagar-Mathur et al., 2008; DaCosta and Huang, 2006; Mahajan and Tuteja, 2005). It is known that the higher levels of proline and soluble sugar were detected in transgenic plants under stress conditions (Hseih et al., 2002; Vannini et al., 2007; Xu et al., 2008). Similarly, our results demonstrated that LeERF1-sn transgenic tomato plants accumulated higher levels of proline and soluble sugar, compared with WT and LeERF1-as tomato plants under drought stress (Figure 4). The increased levels of proline and soluble sugar stabilized membranes and enhanced tolerance to drought stress in LeERF1-sn transgenic tomato plants.

Increasing studies indicated that many ERF transcription factors activated the expression of stress-responsive genes (Huang et al., 2004; Hu et al., 2008). We analyzed the expression of several stress-related genes in WT and *LeERF1* transgenic lines. In contrast to WT and LeERF1-as transgenic lines, LeERF1-sn transgenic plants showed significant activation of several stress-related genes, such as *P5CS*, *LEA*, *ltpg2* and *tdi-65*. For example, as a key gene of proline synthetase, the expression level of P5CS in LeERF1-sn transgenic plants was higher than that in WT and LeERF1-as transgenic lines under drought stress (Kavi et al., 1995). The results indicated that *LeERF1* might activate proline synthetase to enhance tolerance to drought stress in tomato plants. Furthermore, the expression levels of *LEA* encoding a late embryo-genesis protein, *ltpg2* encoding a lipid transfer protein, and *tdi-65* encoding a drought-induced cysteine protease in LeERF1-sn transgenic plants were higher than those in WT and LeERF1-as transgenic lines under drought stress (Wang et al., 2006; Yubero-Serrano et al., 2003; Harrak et al., 2001). These results indicated that *LeERF1* might up-regulate the expression of stress-related genes and resulted in the tolerance to drought stress in LeERF1-sn transgenic tomato plants.

In conclusion, the *LeERF1* gene is an important element for tolerance to drought stress. These results demon-strated that overexpression of *LeERF1* in tomato plants

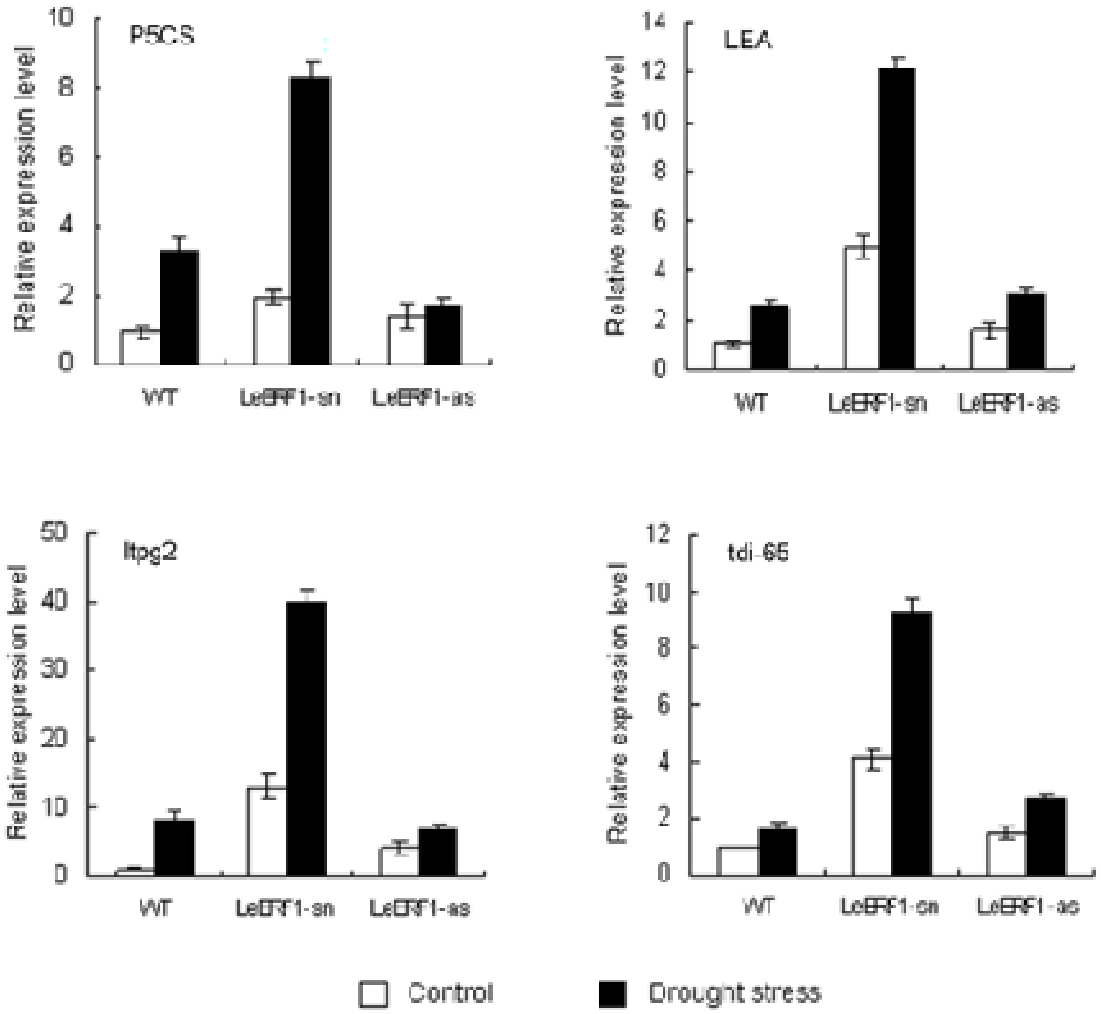

Figure 5. The expression levels of stress-related genes in wild-type (WT), sense-*LeERF1* (LeERF1-sn) and antisense-*LeERF1* (LeERF1-as) tomato plants under control and drought stress conditions. The expression levels of *LEA*, *P5CS*, *ltpg2* and tdi-65 transcripts were assessed by qRT-PCR. The results are the mean ± SD of three individual measurements.

led to the accumulation of osmolytes including free proline and soluble sugar, prevented increased of level MDA and electrolyte leakage, and up-regulated several stress-related genes in tomato plants under drought stress. To further understand the mechanism of the *LeERF1*-mediated signaling pathway in response to stresses, it will be essential to identify the stress-related genes directly activated by *LeERF1*.

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Full Length Research Paper

The effect of gibberellic acid applications on the cracking rate and fruit quality in the '0900 Ziraat' sweet cherry cultivar

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This study was conducted to determine the effects of different gibberellic acid (GA_3) doses (0, 5, 10, 15, 20 and 25 ppm) on the fruit quality and cracking rate in the '0900 Ziraat' sweet cherry cultivar. In this study, different GA_3 doses affected significantly ($p < 0.05$) the most important characteristics of fruit such as fruit weight, fruit firmness and cracking rate determining the marketable value. The lowest and highest fruit weight was 7.95 and 10.02 g in control and 15 ppm GA_3 treatments, respectively. Similarly, the lowest and highest fruit firmness was found to be 7.45 and 9.63 N in control treatment and 15 ppm GA_3 treatments, respectively. In addition, cracking index of 5.60 and 25.50% was obtained for 20 ppm GA_3 and control treatments, respectively. It was also found that GA_3 treatments delayed the harvest date for 3 - 4 days and increased the fruit weight by 10.71% in comparison with the control. Furthermore, the application of GA_3 decreased the fruit cracking rate by 77.80% in comparison with the control. Fruit colour values were also affected by GA_3, application, and brighter and darker red coloured fruits were obtained.

Key words: Sweet cherry, gibberellic acid, cracking index, fruit quality.

INTRODUCTION

There are appropriate climate conditions in Turkey for growing cherry (*Prunus avium* L.). For this reason, it is among the leading countries in the world for sweet cherry production (Kaşka, 2001, Özçağıran et al., 2005, Vursavuş et al., 2006). Turkey has about 18.04% of the world's sweet cherry production that amounts to 338.361 tons (Anonymous, 2008). Turkey is also the biggest exporter country and meets 23.64% of the world's sweet cherry exports of 57.019 tons (Anonymous, 2007). The most important factor in the increase of sweet cherry export is that the significant '0900 Ziraat' sweet cherry cultivar whose fruit is large and firm and fruit pedicles are long, is durable in transportation and has lengthy storage (Kaşka 2001; Aşkın et al., 2008).

The increase in the demand for sweet cherry recently has gradually increased the importance of the storage potential and fruit quality. The fruit quality of the sweet cherry may also be affected by some chemical applications before and after the harvest other than the classic cultural applications (Muskovics et al., 2006). The harvest time may change according to ecologic conditions (Gerçekçioğlu and Polat, 1998) and also the crackings resulting from the rain that happens closer to the harvest time is an another significant factor affecting the quality of the sweet cherries (Jedlow and Schrader, 2005; Cline and Trought, 2007). Particularly, in sensitive varieties, the cracking rate goes up to 90% (Simon, 2006). The cracking that causes the fruit to get easily wrinkled may change according to the genetic characteristics of the varieties (Cline and Trought, 2007). Having a thicker cuticula, the '0900 Ziraat' sweet cherry cultivar increases its resistance against cracking (Demirsoy and Bilgener, 1998; Bilgener et al., 1999; Demirsoy and Bilgener, 2000; Kaşka, 2001). Nevertheless, the losses in the marketable fruit quantity resulting from cracking can

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Table 1. The effect of GA_3 doses on some fruit characteristics in the '0900 Ziraat' sweet cherry cultivar.

Application (ppm)	Fruit weight (g)	Fruit width (mm)	Fruit length (mm)	Shape index	Firmness (N)	Seed weight (g)	Seed width (mm)	Seed length (mm)	Fruit flesh/pit ratio	Peduncle length (mm)
Control	7.95c	24.86	23.60b	0.95	7.45c	0.31c	8.53	11.14	24.38	47.04
5	8.76bc	24.69	24.29ab	0.98	8.56ab	0.32bc	8.54	11.09	26.64	47.61
10	9.42ab	24.79	24.30ab	0.98	9.03ab	0.34bc	8.62	11.28	26.71	48.48
15	10.02a	25.30	24.47ab	0.97	9.63a	0.36a	8.80	11.25	27.13	49.45
20	9.63ab	25.05	25.32a	1.02	8.95ab	0.34ab	8.61	11.30	27.05	49.05
25	9.71a	25.06	24.49ab	0.98	8.16bc	0.34a	8.77	11.25	26.62	49.26

With each column, values followed by the same letter are not significantly different at P = 0.05 level according to Duncan's multiple range test.

be seen in this cultivar.

For this reason, applications that increase the resistance against cracking are being applied to the varieties that are preferred by the consumers and have good fruit quality. One of the leading applications in the world is chemical substance applications such as gibberellic acid (GA_3), Ca, K, NH_4, NO_3, CH_2 and N_2 (Demirsoy and Bilgener, 1998; Clayton et al., 2003). Another aim of such applications is also to delay the harvest date and increase the marketing time of those cherries that can not be stored for a long time.

Therefore, this study was conducted to determine the effects of gibberellic acid applications in different doses on the cracking rate and some fruit quality characteristics in the '0900 Ziraat' sweet cherry cultivar, known world-wide as the 'Turkish Sweet Cherry'.

MATERIALS AND METHODS

In this study, the '0900 Ziraat' cultivar grafted onto a 14-year-old wild cherry (*Prunus avium* L.) rootstock in Uluborlu located in Isparta province was used. GA_3 doses were applied to the trees at the rate of 0 (control), 5, 10, 15, 20 and 25 ppm. The applications were made when the fruits were at the straw-yellow stage (about 30 - 40 days prior to the harvest). Only water was applied to the control trees. Starwet was used as an adhesive spreader. The fruits picked at the harvest time were immediately transported to the post harvest physiology laboratory in ice containers. The measurement of 30 fruits were determined using digital caliper for fruit, seed and pedicel length (mm), fruit and seed width (mm). Fruit and seed weight (g) were determined by a digital scale sensitive to 0.01 g. Fruit firmness (Newton) was measured by digital penetrometer (Lloyd LF Plus). The rates of soluble solid content (SSC) were measured by refractometer (Atago, Japan) and titrable acidity (TA) by titration with 0.1 N NaOH and expressed in percent of citric acid per 100 ml of juice, and pH values were measured with a digital pH meter (Hanna HI221) in three parallel ways. The colour of the fruit was determined with a colorimeter (Minolta Chroma Meter CR-100) using the L* a* b* scale. The cracking index was calculated according to the method of Bilgener et al. (1999). The fruits were harvested in early morning and transported to the laboratory for analyses. The sensibility to fruit cracking was evaluated by immersion of 50 fruits in distilled water at 20°C in 2 L pots for 6 h. The cracked fruits were counted and taken out of the water at intervals of 2 h; the others were immersed in the water again. This practice was repeated 3 times. The cracking index was calculated according to the formula:

$$\text{Cracking index} = (5a + 3b + c) \times 100/250$$

Where, a: Number of cracked fruit after 2 h, b: after 4 h and c: after 6 h; multiplication factor of 5, after 2 h, 3, after 4 h and 1, after 6 h; maximum cracking: 50 x 5 = 250; total number of fruits immersed: 50; multiplication factor of the cracked fruits after the first 2 h was 5.

The experiment was based on randomized blocking pattern as 3 replications and was assigned as one tree at each replication. The data were statistically analyzed using the Statistical Package for the Social Sciences (SPSS) software program according to the variance analyses method. The differences between applications were tested using the Duncan multiple range test.

RESULTS AND DISCUSSION

GA_3 applications significantly ($p < 0.05$) increased the fruit weight in the '0900 Ziraat' sweet cherry cultivar. The heaviest fruits (10.02 g) were obtained from the application of 15 ppm GA_3 with a 10.71% increased in weight when compared to control (Table 1). Horvitz et al. (2003) showed that GA_3 applications delayed the harvest time and significantly contributed to the weight increases. While in this study, a significant difference between applications in terms of fruit width was not observed, the difference in fruit length was significant ($p < 0.05$). The tallest fruits were obtained from the dose of 20 ppm (25.32 mm) (Table 1). The highest flesh/pit ratio in the study was obtained from 15 ppm GA_3 application. The difference in terms of shape index between the applications was not significant ($p < 0.05$). The fruit size is one of the most important quality parameter in sweet cherry. The demand for sweet cherry is optimum at 10 g fruit weight and 25 mm fruit width as an average (Horvitz et al., 2003). For this reason, as the big fruits are much more flesher, they are preferred more by the consumers (Özkaya et al., 2006; Horvitz et al., 2003; Cline and Trought, 2007). Besides this, it was reported in previous studies that GA_3 applications increased cell division and elongation and had a positive effect on fruit sizes (Neil and Cathey, 1960; Looney, 1996; Basak et al., 1998;

Table 2. The effect of GA_3 doses on fruit chemical characteristics and colour characteristics in the '0900 Ziraat' sweet cherry cultivar.

Application (ppm)	SSC (%)	pH	Titrable acidity (% citric acid)	L*	a*	b*
Control	16.48	3.88	0.64a	21.79c	16.36b	3.65c
5	16.28	3.92	0.61bc	29.14ab	24.42a	7.76a
10	16.35	3.87	0.59c	27.31b	20.24ab	5.50b
15	16.10	3.89	0.63ab	27.73b	21.20a	6.07ab
20	16.23	3.85	0.60c	30.00a	24.36a	7.59a
25	16.24	3.86	0.58c	28.44ab	22.10a	7.71a

SSC, soluble solid content. With each column, values followed by the same letter are not significantly different at P = 0.05 level according to Duncan's multiple range test.

Bilgener et al., 1999; Clayton et al., 2003; Horvitz et al., 2003; Usenik et al., 2005; Cline and Trought, 2007). For fruit firmness, there was a statistically signi-ficant difference in the GA_3 applications ($p < 0.05$). The firmest fruits have been determined from the dose of 15 ppm (9.63 N). The consumer tendencies show that a firm sweet cherry is preferred much more than a soft sweet cherry (Esti et al., 2002; Kappel and Mac Donald, 2007; Chauvin et al., 2009).

There are genetic differences between the varieties in terms of fruit firmness. GA_3 applications also had a positive effect on increasing the firmness of the sweet cherry fruits (Kappel and MacDonald, 2002; Blazkova et al., 2002; Clayton et al., 2003; Özkaya et al., 2006). In this study, GA_3 applications increased seed weight, width and length (Table 1). This increase in the seed weight was statistically significant ($p < 0.05$). It was previously found that GA_3 increased both the fruit and seed sizes (Sabır, 1995; Sütyemez, 2000). In this study, it was determined that GA_3 applications relatively increased the fruit peduncles in comparison with the control. However, this effect was not statistically significant (Table 1). The sweet cherry becomes deformed more quickly than in other fruits because of the peduncle's fast water consum-ption and high respiration (Horvitz et al., 2003). For this reason, a long peduncle is a desired feature in sweet cherry fruits as it extends its shelf-life. It was also reported in previous studies that GA_3 had a positive effect on extending the peduncle of the fruit (Patterson and Kupferman, 1983; Sabır, 1995).

No significant differences were found between GA_3 applications in terms of SSC and pH values in the '0900 Ziraat' sweet cherry cultivar. Regarding acidity, statistical difference was found between the applications ($p < 0.05$). In this study, GA_3 applications had a decreasing effect on the acidity value, the highest acidity was obtained in the control application (0.64%) and the lowest acidity was obtained in the dose of 25 ppm GA_3 (0.58%) (Table 2). While some studies showed that GA_3 did not have any significant effect on SSC and acidity (Sabır, 1995; Bilgener et al., 1999; Horvitz et al., 2003; Clayton et al., 2003; Usenik et al., 2005; Cline and Trought, 2007), some studies showed that GA_3 affected the SSC and acidity values positively or negatively (Sütyemez, 2000; Kappel and MacDonald, 2002; Özkaya et al., 2006; Kappel and MacDonald, 2007).

A statistically significant difference was found between GA_3 applications in colour values (L*, a*, b*). L* value varied between 21.79 (control) and 30.00 (20 ppm). The highest a* and b* values were found in 5 ppm GA_3 application (24.42 and 7.76, respectively) (Table 2). As a result, brighter and darker red coloured fruits were obtained. Fruit skin colour is important for both fruit quality and fruit maturity (Usenik et al., 2005; Romano et al., 2006; Gunes et al., 2006; Chauvin et al., 2009). The dark colour in fruits is accepted as an important charac-teristic in terms of the amount of antioxidant substances they contain which has a protective effect against cancer and heart diseases (Romano et al., 2006). For this reason, GA_3 applications enable the sweet cherry fruits to have much more red colour, therefore they are preferred more by the consumers (Esti et al., 2002; Özkaya et al., 2006; Romano et al., 2006; Chauvin et al., 2009).

In this study, the preharvest GA_3 applications decreased the cracking index in the fruits. The least cracking in the fruit was found in the 20 ppm GA_3 application. The most cracking was found in control (25.5%) (Table 3). These results are in line with previous studies which showed that GA_3 applications decreased cracking in the fruits (Bilgener et al., 1999; Demirsoy and Bilgener, 2000; Horvitz et al., 2003; Usenik et al., 2005; Cline and Trought, 2007).

The cracking in sweet cherry occurs when it absorbs rain water in the epiderm. In addition, the speed of water intake, skin permeability and fruit turgor have all been considered as factors that may increase the incidence of cracking (Bilgener et al., 1999). Besides the genetic sensitivity of the varieties to cracking, the type of the soil for growing fruit and its humidity affects the incidence of cracking (Simon, 2006). This cracking in the fruit skin causes fruit loss and presents a serious risk for the grower (Demirsoy and Bilgener, 2000; Simon, 2006; Cline and Trought, 2007). It is reported that GA_3 appli-cations positively affect the thickness of the epiderm and cuticula layer of the fruit and increases the resistance against cracking (Cline and Trought, 2007).

Table 3. The effect of GA_3 doses on fruit cracking rate in the '0900 Ziraat' sweet cherry cultivar.

Application (ppm)	Cracking index (%)	Decrease % in comparison with control
Control	25.50a	100.00
5	16.90b	66.60
10	7.40e	29.20
15	8.90d	34.90
20	5.60f	22.20
25	10.50c	41.07

With each column, values followed by the same letter are not significantly different at P = 0.05 level according to Duncan's multiple range test.

In addition, GA_3 decreases the cracking in fruit by delaying maturity time of fruits and getting over critical rain period (Usenik et al., 2005). As a result, it was observed that the preharvest GA_3 applications had a positive effect on the sweet cherry fruit quality characteristics. Particularly, they delayed the harvest date of 3 - 4 days which caused an increase in the weight ratio of 10.71%, increased the fruit firmness, helped to obtain darker red coloured fruits with a higher acceptance to the consumer and decreased the cracking rate on the surface of the fruit.

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Full Length Research Paper

Effects of strip and full-width tillage on soil carbon IV oxide-carbon (CO_2-C) fluxes and on bacterial and fungal populations in sunflower

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In strip tillage system, planting lines are cultivated while the inter-row spaces are left undisturbed. The objective of this study is to determine the effects of strip tillage and full-width tillage treatments on soil carbon IV oxide-carbon (CO_2-C) fluxes, bacterial and fungal populations in growing period of sunflower (*Helianthus annus*). A row-crop rotary hoe with C type blades was used to create three strip widths by changing the connection of blades of the rotary hoe on the flanges. Strip widths were 22.5 (T30), 30.0 (T40) and 37.5 cm (T50). The full-width tillage practice (moldboard plow + disc harrow + leveler) gave 100% surface soil disturbance (T100) and was included in the experiment to make comparisons with the strip tillage system. A randomized complete block design with three replications was used. During the growth of the sunflower, periodic measurements of CO_2-C fluxes and bacterial and fungal populations were made. Significant ($p < 0.01$) differences in CO_2-C fluxes, microbial populations, soil bulk density and total porosity were observed between the different tillage systems. Highest CO_2-C fluxes, bacteria populations and total porosity were observed in the full-width T100 application and the lowest values were observed in the T30 treatment during flowering and harvesting periods. Increasing tillage intensity increased soil CO_2-C fluxes and bacteria population, but decreased fungi population and soil bulk density.

Key words: Carbon IV oxide-carbon flux, soil bacteria and fungi, strip tillage, full-width tillage, sunflower.

INTRODUCTION

Soil is an open system and can be a net source of carbon IV oxide (CO_2) released to the atmosphere due to elevated soil organic carbon mineralization caused by disruptive agricultural practices. Soil organic carbon (C) plays an essential role in determining many soil properties and thus, greatly influences the fertility of the soil. The C in organic matter is more resistant to decomposition than the residues from which it is derived. The distribution of the organic C is also changed from that in a plowed soil and stratification often occurs. If soil is not tilled, plant roots and various natural soil processes continue to increase the levels of organic C from the surface of the soil downward (Triplett and Dick, 2008).

The two most important processes affecting C balance of terrestrial ecosystems are photosynthesis of above-ground vegetation and soil respiration. The relationship between soil organic matter production and decomposition determines whether a system is a sink or a source of atmospheric CO_2. Soil can function as a net sink for sequestering atmospheric CO_2 under appropriate soil and crop management systems, thus reducing atmospheric CO_2 (Paustian et al., 1992; Lal et al., 1995). In contrast, soil respiration can release large quantities of CO_2–C from the soils to the atmosphere. Changes in land use and soil management practice (tillage, use of fertilizers, organic residues and pesticides) are largely responsible for increases in atmospheric CO_2 from terrestrial ecosystems because they induce transformation of soil organic

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Abbreviations: ST, Strip tillage; **EC,** electrical conductivity; **CFU,** colony forming units; **PBS,** phosphate-buffered saline solution; **SEA,** soil extract agar; **DPA,** dextrose-peptone agar.

C to atmospheric CO_2 (Bouwman, 1990).

Different tillage systems using common soil tillage implements (e.g. disk harrow, reversible disk plow, rotary tiller and chisel plow) produced different levels of CO_2 fluxes (La Scala et al., 2001). Measurements of soil CO_2 flux for different tillage treatments and cropping systems are important for identifying management practices that may affect this flux and thus affect the global C balance (Houghton et al., 1983; Post et al., 1990). The CO_2 loss is related to volume of soil disturbed by the tillage operation and maximum CO_2 loss was observed for moldboard plow, with various conservation tillage tools losing only 30% of that lost by the plow (Reicosky, 1999). The use of the moldboard plow creates two major soil effects: (1) Loosening and inverting the soil to allow rapid CO_2 loss and oxygen entry and (2) incorporation or mixing the residues, which leads to enhanced microbial attack (Reicosky and Lindstrom, 1995). Moldboard plowing at increasing depths has generally resulted in reductions in soil C pool sizes when compared to grasslands or native ecosystems (Potter et al., 1999; Soussana et al., 2004).

Strip tillage is a form of conservation tillage that involves cultivation of narrow bands, or strips in the row area, separated by bands of undisturbed soil. Strip tillage has the potential advantages of providing a suitable seedbed for various row crops establishment while leaving surface residues in the inter-row area to reduce soil erosion (Peterson et al., 1996; Wilhoit et al., 1990). Strip tillage is hypothesized to decrease the amount of CO_2 loss relative to plowing. Only a relatively small amount of CO_2 was detected immediately after strip tillage and this amount was related to the volume of soil disturbed (Reicosky, 1999).

Intensive tillage often involves using the moldboard plough to invert the soil followed by secondary tillage tools to break up and homogenize soil clods. One effect of such intensive tillage is to increase compactness, which decreases space between pores, thereby changing the pathway for CO_2 diffusion (Sanchez et al., 2002). Tillage accelerates soil CO_2 emission by improving soil aeration, disaggregating soil, increasing the contact between soil and crop residue, and speeding organic C decomposition (Logan et al., 1991; Angers et al., 1993; Kern and Johnson, 1993; Al- Kaisi and Yin, 2005).

Soil microbial biomass C and soil enzyme activities are affected by no-tillage in a manner similar to that of organic C (Franzluebbers, 2002; Dick, 1984). When the crop production system is changed to less intensive tillage system, the microbial biomass and the biologically active C and N pools respond rapidly and the changes are more easily measured than changes in total C and N. No-tillage changes both the profile distribution of biological activity and the biological community itself, with fungi becoming more dominant under no-tillage (Six et al., 2006). Fungal dominated soil communities may enhance C storage and slow soil organic matter turnover due to both the fungal alteration of soil physical properties and to fungal physiology (Nakas and Klein, 1979; Tisdall and Oades, 1982). Ecosystems with soils dominated by fungi thus reduce CO_2-C flux and sequester more C than systems with lower fungal abundance (Six et al., 2006).

Soil organic matter is a resource for soil biota and there is a strong relationship between the abundance of soil organisms and the content of organic matter (Wardle et al., 2001; Nakamoto and Tsukamoto, 2006). Many soil organisms receive benefits because of a reduction in soil disturbance and an increase in surface crop residues.

The purpose of this study is to evaluate the effects of different widths strip tillage system and full-width inversion tillage system on soil bulk density, porosity, CO_2-C fluxes and on bacterial and fungal populations, in sunflower (*Helianthus annus*).

MATERIALS AND METHODS

Experimental site

The experiment was conducted at the research farm of Ataturk University (39° 54' N and 41° 13' E, altitude 1883 m), Erzurum, Turkey. The soil on the experimental sites was classified as Ustorthents according to the United States Department of Agriculture (USDA) soil taxonomy (Soil Survey Staff, 1999). The slope of the experiment area was under the 0.5% and there was no erosion problem. Some climatic data relating to the experimental area are provided in Table 1. The initial crop was winter wheat. Wheat was machine harvested from the experimental area at the end of August 2005. Stubble height was left around 10-12 cm. Soil tillage and sunflower planting were performed after wheat harvest during the third week of May 2006.

Experimental design

Plots were arranged in a randomized complete block design with three replications. Each treatment plot was 3 by 30 m in size and plots were separated by buffer areas that were one-half the size of the treatment plots. A strip tillage (ST) system with three different strip widths (22.5 cm) 30%, soil surface disturbance (T30) 30.0 cm, 40% soil surface disturbance (T40) and 37.5 cm, 50% soil surface disturbance (T50) was compared with a full-width inversion tillage system with 100% soil surface disturbance (T100). The three strip widths were achieved by changing the connections of blades of the rotary hoe on the flanges (Figure 1). Tilled zones of 30, 40 and 50% of the field area were obtained by use of two, three or four blades, respectively. The rotary hoe was operated at constant rotor rotational speed of 370 rpm. Tillage in the full-width inversion system (T100) involved the use of a moldboard plow followed by a disk harrow and a soil leveler. The tillage depth was kept constant at 12 cm for the strip tillage systems and 20 cm for the full-width inversion tillage system. The tractor operating speed was 1.5 m s^{-1} by using a DJRVS II speed radar and a DJCMS100 monitor made by Dickey-John (5200 DICKEY-John Road Auburn, IL).

Soil tillage was performed on 16 May 2006. After the seedbed preparation on 19 May 2006, all plots were sown using a four row pneumatic planter, commonly used in sunflower planting in Turkey, with 70 cm inter-row spacing. Urea fertilizer (50 kg N ha^{-1}) and triple super phosphate (70 kg P_2O_5 ha^{-1}) was applied at planting. Weed control was accomplished by hand hoeing twice per month.

Soil sampling and laboratory analysis techniques

Plots which have the same physical and chemical properties were

Table 1. Monthly rainfall means temperature and average relative humidity for 2006.

Month	Monthly rainfall (mm)	Mean temperatures (°C)	Average relative humidity (%)
January	17.8	-11.2	81.6
February	10.9	-5.6	77.0
March	13.4	1.2	73.5
April	77.4	7.2	74.4
May	41.6	11.4	67.3
June	19.2	18.4	56.7
July	20.7	20.3	62.5
August	3.5	22.6	50.9
September	29.2	14.1	60.2
October	90.1	8.6	76.0
November	25.3	-0.1	70.9
December	8.3	-9.8	75.4

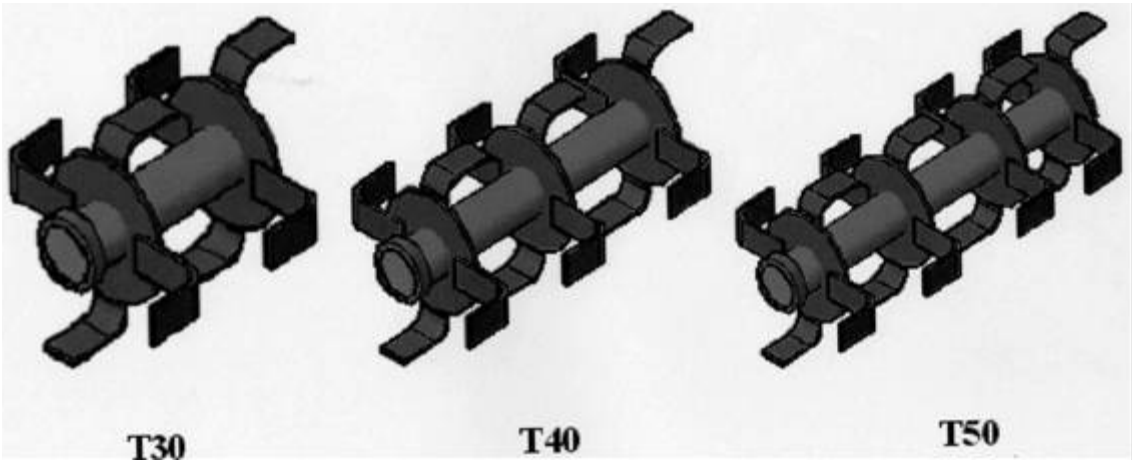

Figure 1. Application of tillage strip width by modification of rotary hoe. T30 = Strip width of 22.5 cm, 30% soil surface disturbance and two blade; T40 = strip width of 30.0 cm, 40% soil surface disturbance and three blade; T50 = strip width of 37.5 cm, 50% soil surface disturbance and four blade.

sampled for soil at three different sunflower growing periods that included planting (June 20), flowering (August 10) and dough maturity (September 12). Depth of sampling was 0 - 20 cm for initial soil chemical and physical properties and samples were removed from the field using a 5 - cm diameter borer. Separately, on 10 May, before sunflower sampling, the soil was sampled and this sample was used as an untreated control. Three soil samples were collected from Ap horizon of each plot in each three period every day during a couple of weeks. Collected soil samples were sieved through a 2-mm mesh opening on the field and brought to the laboratory for initial chemical, physical and microbial analysis. After soil tillage systems application, depth of sampling was 0 - 12 cm for strip tillage and 0 - 20 cm for full-width inversion tillage system for soil bulk density, porosity, CO_2-C analysis and soil microbial population analysis at the sowing, flowering, and harvesting period and plant growth.

Soil organic C was determined by the Smith-Weldon method (Tiessen and Moir, 1993), $CaCO_3$ content was determined using a Schleibler calcimeter (Tee et al., 1993), total nitrogen (N) was determined by using the micro Kjeldahl method (McGill and Figueiredo, 1993), soil pH was determined by using a glass electrode pH meter (1:2.5, soil: water), exchangeable cations and cation exchange capacity were determined by atomic absorption spectrophotometer after the method of Handershot et al. (1993), available P was determined by the Na_2CO_3 extraction method (Olsen and Sommers, 1982), field water capacity was determined by the tensiometer method (Topp et al., 1993), soil texture b was determined by the Bouyoucus hydrometer method (Shieldrick and Wang, 1993), electrical conductivity (EC) was determined by using an EC meter according to the method of Janzen (1993), and soil bulk density and particle density were determined by the core method and total porosity was calculated from bulk density and particle density (Blake and Hartge, 1986). Measured chemical and physical properties of the experimental site soil are shown in Table 2.

Microbial population analysis

Determinations of viable microbial bacteria and fungi counts were carried out at four different growing periods (initial, sowing, flowering and harvesting) of sunflower. Gentle tapping separated the rhizosphere soil adhering to the root, and this recovered soil was analyzed the same day.

Table 2. Some initial chemical, physical and microbiological properties of the experimental site.

Soil property	Value
pH (1:2.5)	7.16
Organic matter, g kg^{-1}	2.29
Lime ($CaCO_3$), g kg^{-1}	1.11
Total N, g kg^{-1}	0.14
Plant Available P mg kg^{-1}	4.71
Exchangeable cations, cmol kg^{-1} soil	
Ca	22.49
Mg	7.27
K	2.61
Na	0.29
Microelements, mg kg^{-1}	
Fe	5.97
Cu	1.79
Zn	1.17
Mn	9.52
Cation exchange capacity, cmol kg^{-1}	35.0
Electrical conductivity, dS m^{-1}	0.65 x 10^3
Salt, %	0.016
Field capacity at 1/3 atm, g kg^{-1}	26.7
Wilting capacity at 15 atm, g kg^{-1}	15.4
Particle size distribution, g kg^{-1}	
Sand	32.3
Silt	44.1
Clay	23.6
Bulk density, Mg m^{-3}	1.51
Porosity, vol. %	42.25
Microbiological properties	
Number of bacteria, CFU* g^{-1} soil	2.55 x 10^7
Number of fungi, CFU g^{-1} soil	2.45 x 10^5
Total C respired as CO_2, mg m^{-2} h^{-1} (CO_2-C)	3.2 (0.27 Mg C ha^{-1} y^{-1})

*CFU, Colony-forming units.

Culturable bacteria and fungi (colony forming units, CFU) were enumerated by the spread soil dilution plate method. For this method, each 10 g soil sample was homogenized in 100 ml phosphate-buffered saline solution (PBS, 0.15 M potassium phosphate, 0.85% NaCl, pH 7.0). The sample was centrifuged for 7 min at 250 ×*g* and the supernatant decanted into a sterile flask. The pellet was resuspended and washed twice by centrifugation in sterile PBS. All fractions were pooled in a sterile flask and serially diluted (10^6 – 10^7) in PBS (McDermott, 1997). For bacteria, 0.1 ml of each dilution of the series was placed onto a Petri dish with soil extract agar (SEA) (Ogram and Feng, 1997), for fungi, 0.1 ml of each dilution was placed onto a Petri dish with dextrose-peptone agar (DPA). To inhibit fungal growth during bacterial measurements, 30 mg l^{-1} cycloheximide was added to the SEA. To inhibit bacterial growth during fungi measurements, 30 mgl^{-1} streptomycin were added to the DPA. (Alef, 1995b). Three replicate dishes were made for each dilution. The agar plates were aerobically incubated at 30°C for 7 days to obtain bacterial counts and at 25°C for 7 days for fungi counts. After the incubation period, the CFU of the bacteria and fungi developed on the respective agar plates were enumerated using an automated colony counter. The averaged CFU per gram of oven-dried soil was calculated for each soil sample (Canbolat at al., 2006; Madigon and Martinko, 2006) and results are reported in Table 2.

CO_2-C analysis

The estimation of CO_2-C evolved from soil during incubation was conducted using a closed system. CO_2 was trapped in a NaOH solution, which was then titrated with HCl. For this experiment, 50 g sieved soil was placed in a beaker and the beaker placed in the bottom of a one-liter jar. A total of 25 ml NaOH (0.05 M) was pipetted into the jar and the jar was immediately sealed to make it airtight using a rubber ring and two crossing pegs. Other jars with NaOH (0.05 M) but without soil were used as controls. The jars were incubated up to 3 days at 25°C. At the end of incubation, the beakers were removed from the jars and the external surface of the beaker was rinsed with CO_2-free water and this water was added to the the NaOH solution in the jar. Five mille of $BaCl_2$ solution (0.5 M)

Table 3. Effect of soil tillage intensity on soil bulk density, porosity and CO_2-C fluxes at sowing, flowering, and harvesting periods of sunflower (*Helianthus annus*) and Duncan's multiple range tests.

Tillage system	Sowing period			Flowering period			Harvesting period		
	Bd	P	CO_2-C fluxe	Bd	P	CO_2-C fluxe	Bd	P	CO_2-C fluxe
T30	1.52a	43.46d	3.48 (0.301†)c*	1.49a	42.10d	4.36 (0.377)c	1.48a	40.15c	4.90 (0.423)c
T40	1.45ab	45.15c	3.51 (0.303)c	1.45ab	44.85c	4.40 (0.380)c	1.43ab	44.08b	4.96 (0.429)b
T50	1.39b	47.08b	3.64 (0.314)b	1.38b	46.53b	4.56 (0.394)b	1.38b	47.15ab	5.02 (0.434)b
T100	1.33c	49.62a	3.72 (0.321)a	1.34c	48.93a	4.65 (0.402)a	1.34c	48.23a	5.08 (0.439)a
Average	1.42A	46.32A	3.58 (0.309)C	1.41A	45.60B	4.49 (0.387)B	1.40A	44.90C	4.99 (0.431)A

*Means with the same letter are not statistically significant ($p < 0.01$); Bd, bulk density (Mg m^{-3}); P, total porosity (vol. %); CO_2-C fluxes (mg m^{-2} h^{-1}); †variables in parentheses Mg C ha^{-1} y^{-1} are indicator of the stability of the soil organic carbon in the soil; T30 = strip width of 22.5 cm, 30% soil surface disturbance, two blade; T40 = strip width of 30.0 cm, 40% soil surface disturbance, three blade; T50 = strip width of 37.5 cm, 50% soil surface disturbance, four blade; T100 = conventional tillage (moldboard plow + disk harrow +l eveler), %100 soil surface disturbance.

was then added to the NaOH solution along with some drops of phenolthelein indicator. The unreacted NaOH was titrated with standard HCl (0.5 M) with continuous stirring until the color changes from red to colorless. CO_2 that evolved from the soil during the exposure of alkali was calculated using the formula reported by Anderson (1982) and Alef (1995a).

Statistical analysis

Analysis of variance (ANOVA) was used to evaluate the significance of each treatment on soil properties and CO_2 fluxes and on bacterial and fungal populations. Comparison of means was performed, when the F-test for treatment was significant at the 5% level, using Duncan's multiple means tests.

RESULTS AND DISCUSSION

CO2 Fluxes

Average CO_2-C fluxes as affected by tillage treatments and time periods were found to be significantly ($p < 0.01$) different. The highest CO_2-C fluxes from soils in sowing, flowering and harvesting period were observed for T100 system (3.72, 4.65, 5.08 mg C m^2 h^{-1}, respectively). The minimum CO_2-C fluxes were observed for the T30 system at all periods (3.48, 4.36, 4.90 mg C m^2 h^{-1}). The CO_2-C fluxes increased with increasing strip widths. Among the strip widths, the highest fluxes were observed for the T50 systems and the lowest for the T30 systems (Table 3). The CO_2-C fluxes from the T30, T40 and T50 treatments were not statistically ($p < 0.05$) different. The same tendency was observed for all sampling periods.

The highest CO_2-C fluxes were observed at the harvesting period (during the summer period) and the minimum fluxes were observed at the sowing period. At the harvesting period, plant root growth, soil biomass and soil microbial activity had increased and these increases contribute to increased CO_2-C fluxes. Increasing strip tillage width increased CO_2-C fluxes at all sampling periods. Carbon dioxide fluxes from soil are due to the decomposition of organic material by microorganisms and root respiration. Tillage increased the rate of organic C decomposition. Data of the stability of the soil organic carbon in the soil are shown in parenthesis as Mg C ha yr^{-1} in Table 3. These measurements showed that carbon is more stable in the strip-tilled than in the T 100 plots.

Average bulk density and total porosity of the soil as affected by tillage treatments and plant growing periods were found to be significantly ($p < 0.01$) different. Total porosity of the soil increased with increasing strip widths, while bulk density of the soil decreased. The highest bulk density was observed in T30 treatment and the lowest bulk density was observed in T100 treatment at the sowing, flowering and harvesting period of plant growth (Table 3).

Changes in land use and soil management practice (tillage) also induce changes in soil organic C levels and CO_2-C fluxes (Bouwman, 1990; Al- Kaisi and Yin, 2005, Logan et al., 1991). T100 treatment caused the highest CO_2-C fluxes because tillage loosens the soil, increases the exposure of soil organic matter and speeds up organic matter oxidation (Reicosky and Lindstrom, 1993).

Soil tillage is among the important factors affecting soil physical properties (Khurshid et al., 2006). The tillage treatments affected the soil physical properties, especially when similar tillage system has been practiced for a longer period (Jordhal and Karlen, 1993, Mielke and Wilhelm, 1998). Structure of the Ap horizon is largely influenced by soil tillage system and the implements used for tillage (Lal, 1991). All tillage tools reduced the bulk density and raised porosity to the depth of tillage (Ferreras et al., 2000). In our study, we observed that strip tillage and a full-width inversion tillage system effected soil bulk density and soil porosity.

Bacterial and fungal population

The effects of soil tillage systems (between the strip tillage and full-width inversion tillage) on average bacterial numbers were statistically significant ($p < 0.05$). Similar results were observed in different growing periods. The

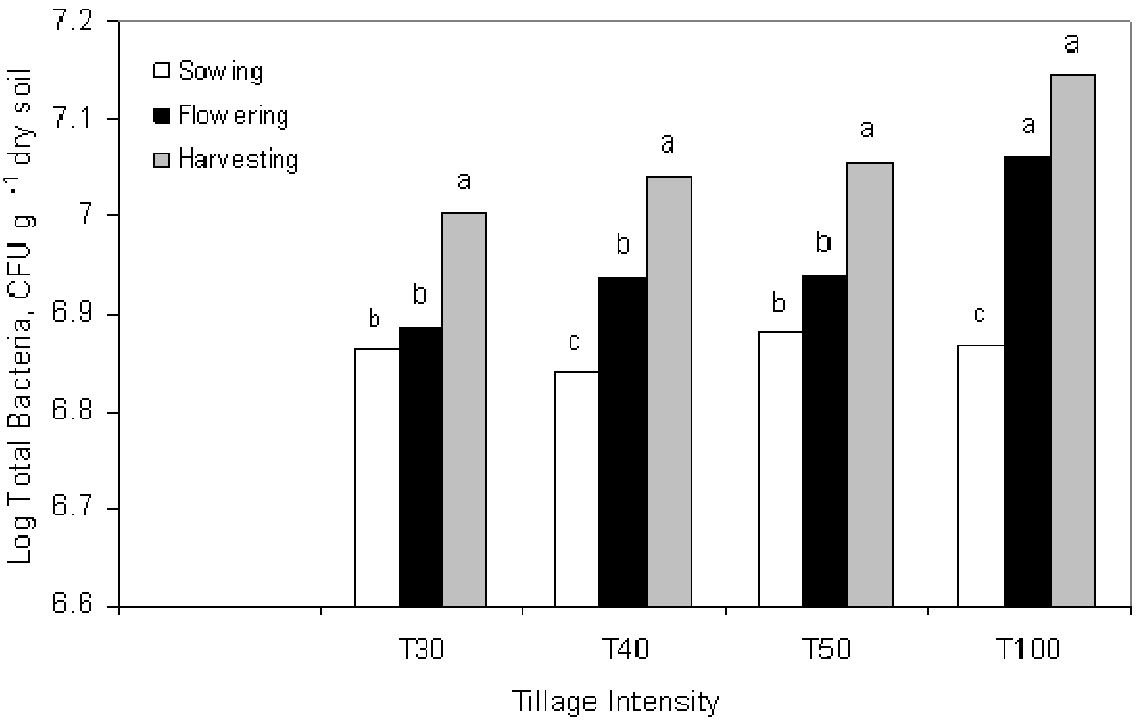

Figure 2. Effect of soil tillage intensity on soil bacteria populations at sowing, flowering and harvesting periods of sunflower (*Helianthus annus*). T30 = Strip width of 22.5 cm, 30% soil surface disturbance and two blade; T40 = strip width of 30.0 cm, 40% soil surface disturbance and three blade; T50 = strip width of 37.5 cm, 50% soil surface disturbance and four blade and T100 = conventional tillage (moldboard plow+disk harrow+leveler), %100 soil surface disturbance.

highest bacterial numbers were observed for the T100 system at flowering and harvesting periods (7.06 and 7.14 CFU g^{-1} dry soil), respectively. Among the strip tillage systems, the T30 plots supported the lowest bacterial numbers (6.88 and 7.00 CFU g^{-1} dry soil) and the T50 plots supported the highest bacterial numbers (108.6 and 142.6 CFU g^{-1} dry soil) at flowering and harvesting periods, respectively (Figure 2).

Similar results were observed for fungi population. The effects of soil tillage systems (between the strip tillage and full-width inversion tillage) and growing periods on average fungi populations were statistically significant ($p < 0.05$). Among the strip tillage systems, the highest fungi populations were observed in T30 system at flowering and harvesting periods (7.13 and 7.30 CFU g^{-1} dry soil), respectively, and T100 supported the lowest fungi numbers (6.94 and 7.11 CFU g^{-1} dry soil). The highest average fungi populations were observed in harvesting period (7.30 CFU g^{-1} dry soil) (Figure 3).

As tillage intensity increased, similar to the CO_2-C fluxes, the population of bacterial and fungal increased. The T100 tillage system produced the highest fungi and bacterial populations when compared with the three strip tillage treatments. Although microbial activity is strongly dependent on plant, soil and climatic conditions, soil tillage intensity had a definite effect on microbial activity (Figures 2 and 3).

Among the sampling periods, the harvesting period supported higher bacterial and fungi populations when compared with the other two periods. At both flowering and harvesting periods, the highest bacterial populations were observed for the T100 system and minimum values were observed for the T30 system. However, the highest fungi populations were observed for the T30 system and minimum values were observed for the T100 system (Figures 2 and 3). Tillage decreases soil organic matter (Gebhart et al., 1994), and when the crop production and tillage systems change, the microbial biomass and the biologically active C and N also rapidly change (Six et al., 2006). Tillage causes immediate changes in microbial community structure, but little concomitant change in total microbial biomass (Jackson et al. 2003). The cumulative effect of tillage and many cropping rotations has been 30 - 50% decrease in soil C that causes an undesirable change in soil physical, chemical and biological properties (Reicosky and Archer, 2007). Similar results were obtained in this study.

Conclusion

The present study demonstrated that increased tillage

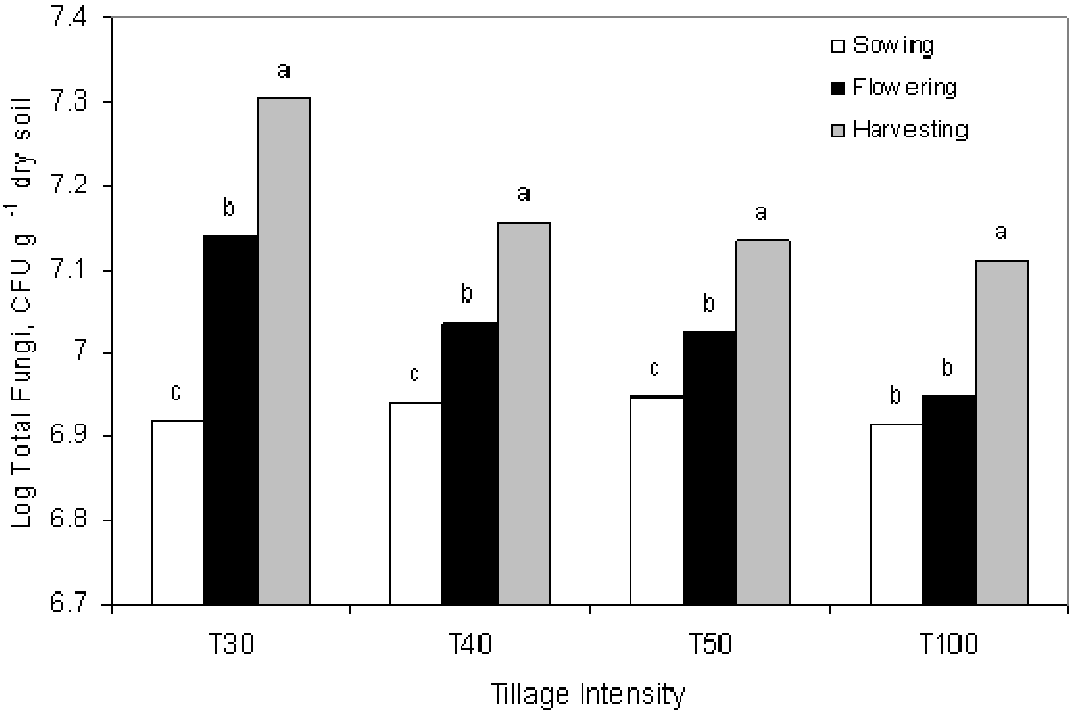

Figure 3. Effect of soil tillage intensity on soil fungi populations at sowing, flowering and harvesting periods of sunflower (*H. annus*). T30 = Strip width of 22.5 cm, 30% soil surface disturbance and two blades; T40 = strip width of 30.0 cm, 40% soil surface disturbance and three blades; T50 = strip width of 37.5 cm, 50% soil surface disturbance and four blades and T100 = Conventional tillage (moldboard plow + disk harrow + leveler), %100 soil surface disturbance.

intensity increased total porosity and CO_2-C fluxes of the soils. Especially, the T100 system exhibited large CO_2-C flux from soil. The increase in CO_2-C flux was related to tillage intensity with the smallest flux associated with the T30 treatment and the largest flux associated with the full-width inversion tillage (T100). This is because increasing tillage intensity disaggregates soil and improves soil aeration. Tillage treatments affected the soil physical and biochemical properties. Increasing tillage tools reduced the bulk density but increased total porosity to the depth of tillage.

The full-width inversion T100 tillage system showed the highest population levels of bacteria and the T30 treatment showed the lowest levels. But, T30 tillage system shoved the highest population levels of fungi and the T100 treatment shoved the lowest levels. As tillage systems change, the microbial biomass and the biologically active C fraction in soil also rapidly change. To preserve soil C and soil microbial populations, reduced tillage systems, such as the strip tillage systems evaluated in this study, can effectively decrease CO_2 emission.

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Full Length Research Paper

Effects of different organic materials and chemical fertilizers on nutrition of pistachio (*Pistacia vera* L.) in organic arboriculture

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This study was conducted under greenhouse conditions to investigate the effects of applied nutrients such as six organic materials (gyttja, alsil, humic acid, sea moss, straw and peat) and two chemical fertilizers (15-15-15, and 20-20-0) with different dosages on nutrient uptaking ability of one-year old and 7 cm long pistachio (*Pistacia vera* L.) trees by analyzing nutrient (mineral) contents of pistachio (*P. vera* L.) leaves. The experiment was designed as randomized block design with four replicates. Even though organic and inorganic materials had different effects on element contents of pistachio (*P. vera* L.) leaves, overall, these materials had statistically significant effects on P, Mg, Na, Mn, and Cu contents of pistachio leaves. The effect of organic and inorganic materials on K, Ca, Zn, and Fe contents of pistachio (*P. vera* L.) leaves was statistically not significant. Besides, inorganic fertilizers increased P content of pistachio (*P. vera* L.) leaves, but this increase caused the decrease of Cu content. High level of P content may be the reason of low uptake of some micro elements such as Cu. Although application of organic and inorganic materials generally increase P uptake, organic materials especially peat, gyttja and sea moss increased nutrient contents of pistachio (*P. vera* L.) leaves. This result is a significant knowledge in pistachio areas because they have low nutrients in there .

Key words: Organic material, chemical fertilizer, *Pistacia vera* L., soil conditioner, arboriculture.

INTRODUCTION

The success in organic production depends upon the systematic approach of soil and plant. Therefore, soil and plant should be planned together and a closed system should be formed by selecting resistant and demand varieties with agro ecosystem. In organic farming, solutions are based on rational, experiential and experimental ecological knowledge (Lammerts et al., 2004).

Anatolia is an important gene source for horticultural crops with varieties which have multiplied numerously during the centuries (Simsek, 2009a; Simsek 2009b). Pistachio (*Pistacia vera* L.) is known as the green almond and is the fifth most important commercial nut crop in the world. The species has been cultivated in Iran (Parsa and Wallace, 1980; Barone, 1997; Mozaffari et al., 2009; Fekri and Gharanjig, 2009; Malakouti and Mozaffari, 2009; Hosseinifard et al., 2010), Turkey (Aydeniz, 1990; Tekin and Güzel, 1992; Kaska, 1995; Tekin et al., 1995; Arpacı et al., 1997; Ak and Fidan, 2009), Tunisia (Ben Mimoun et al., 2005) and Italy (Crescimanno et al., 1987; Barone, 1997; Barone et al., 1998; Caruso et al., 2005) for centuries but more recently, it is being cultivated extensively in USA and Australia (Pillai, 1995; Allemann, 2006).

Ideally, soils should be deep, friable and well-drained, but moisture-retaining (Labavitch et al., 1982). Pistachios can survive on poor, stony, calcareous, highly alkaline or slightly acidy, or even saline soils, and are more tolerant of these conditions than most other nut trees. The tree can survive a wide acidic range, a soil pH between 7.1

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and 7.8 is optimal (Pillai, 1995).

Fertilizer management in pistachio orchards have largely ignored the possibility that mineral demands and tree capacity for nutrients uptake (Weinbaum et al., 1994; Brown, 1995). In the different studies about pistachio, different methods of K applications was tested. The results revealed that K content in the control plots were between 0.98 and 2.06% (Malakouti and Mozaffari, 2009). In Iran, findings suggest the need to apply K fertilizers to old pistachio orchards where exchangeable K might be lower than 200 mg kg^{-1} (Hosseinifard et al., 2010). Recent studies indicated that K fertilization was found to be effective in increasing leaf K status (Zeng et al., 1998). A field experiment was conducted to examine the effects of K fertilization (0, 110, 220, and 330 kg-ha^{-1}) and K sources (K_2SO_4, KCl, and KNO_3) on pistachio. Leaf K concentration was low (<10 g-kg^{-1}) during spring flush. Leaf K concentration increased following K fertilization. There were no significant differences among the K sources in their effects on leaf K concentration. The critical leaf K value for optimal pistachio production was obtained to be 16.9 g-kg^{-1} (Qiupeng et al., 1998). A work was studied to evaluate the effects of potassium fertilization on the leaf mineral content of pistachio tree (*P. vera* L.). Both potassium nitrate and potassium sulfate were used. Concerning the foliar diagnostic, K fertilization increased leaf potassium concentration (0.40 - 0.82%) with no effect observed on N (0.56 -1.34%), P (0.06 - 0.10%) and Mg (0.65 - 0.81%) leaf contents (Mimoun et al., 2005). An experiment included treatments of K. The analysis of leaf samples demonstrated that treatments was increased to K leaf. The high concen-trations of zinc and copper were observed at 13 and 7 mg/kg, respectively. The rate of Na were observed between 0.83 and 0.04%. The effects of treatments on rate of P were not significant (Mozaffari et al., 2009). Other research was conducted to evaluate the effects of some pistachio rootstocks on mineral content of leaves. Mineral contents of cultivars leaves were deter-mined in different levels according to the cultivars (Tavallali and Rahemi, 2007). In the other experiment, results showed that there were no particular significant differences in the foliar concentrations of N, P, Fe, Mn and Zn, whereas, there were significant differences among rootstocks for Mg and K (Barone, 1997). In a different study, mineral contents were recorded in the Sicilian pistachio cultivar. The trees were grown using standard cultural practices in a fine sandy soil and analyzed for the main plant mineral elements. There were no particular significant differences in the foliar concen-trations of N, P, Fe, Mn and Zn, whereas, there were significant differences among the rootstocks for Mg and K. An antagonistic effect was detected between these last two elements, with a highly significant negative correlation between K and Mg (Barone et al., 1998). Zinc deficiency of pistachio trees was characterized visually as 'little leaf', analytically by low levels of zinc, and internally by increases in N, P and total soluble N (Durzan, 1995).

To study the effects of phosphorus levels (0, 50 and 100 P mg kg^{-1}) and salinity levels (0, 1000 and 2000 mg NaCl kg^{-1}) on the chemical composition of pistachio seeding, an experiment was conducted. According to results of this study, adding of P significantly decreased Na concentration in the all of pistachio organs, and increased P, K, Ca and Mg concentrations in leaf and root. Adding of P decreased Fe, Mn and Cu concentrations in leaf, stem and root. Increasing salinity decreased K concentration and increased Ca concentration in stem and root. Application of pistachio waste increased P, K, Ca, Fe, Zn, Mn and Cu concentrations in leaf, stem and root and this increase was different in several organs (Fekri and Gharanjig, 2009). To find the most appropriate nutrient solution for the growth of different pistachio, seedlings were grown in a glasshouse. Treatments included 0, 1/8, 1/4, 1/2, 1, 2, 4, 8, and 10 strength Hoagland solution. Highest growth of aerial and root portions were associated with dilute as 1 strength nutrient solution or lower (Parsa and Wallace, 1980). The ability of the rootstocks to take up nutrients from the soil differed significantly (Crescimanno et al., 1987).

In Turkey, pistachios are grown usually on produced field condition seedlings rootstocks. This seedlings grow very slowly on dry conditions; making seedlings to be grafted 5 - 7 years after transplanting. Potted seedlings have strong root system that grow quickly and adapt easily to orchard soil. After planting, budding is applied in autumn years or the next spring and fruit production begins after 4 - 5 years (Arpacı et al., 1997).

In the Southeastern part of Turkey, pistachios are grown in reddish-brown soil. Approximately, one third of the soil is shallow. Most of the soils are loamy texture, of low salinity and have a pH medium status to extreme alkalinity (8.1 - 8.7). Lime content varies from high to very high. The organic matter and available phosphorus contents of these soils are low and potassium and magnesium content generally sufficient. It was seen that there was deficiency in phosphorus and zinc. However, according to the limit values given for pistachio, there are no major problem with zinc. The iron value was at the lowest limit. There was no problem with copper, calcium and boron (Tekin et al., 1995). In the southeast Anatolia region, it was reported that while pistachio trees were lacking especially nitrogen and phosphorus, the other macro-nutrients such as calcium, magnesium, and potassium were in sufficient amounts, but micronutrient levels might be a problem in the future. Soil organic matter was less than 2 in 66% of pistachio orchards, P was insuf-ficient, 55.2% of the orchards. While all of the top soils had sufficient K, 18% of subsoils had deficiency of it. The 53.3% of pistachio orchards was never fertilized in the region (Tekin and Güzel, 1992).

Besides, nutrient contents of the trees or leaves in the Southeastern part of Turkey region were: N (< 2%), P (0.32 - 1.54%), Fe (90 - 170 ppm), Mn (20 - 115 ppm), Cu (> 10 ppm), and Zn (25 ppm) (Aydeniz, 1990). A study

was conducted to search plant nutrition content of different parts of pistachio cultivars. As the results of the analysis of leaf samples showed, nitrogen, phosphorus and calcium contents were not sufficient, while potassium, magnesium, iron and copper contents of the samples were sufficient. Also it was determined that zinc level is good enough in fruitful pistachio trees, but insufficient in unfruitful trees. Plants could not utilize the elements in the soil because of high level of Ca, low water content and high temperature (Ak and Fidan, 2009).

Various organic based fertilizers were extensively used in agriculture to compensate nutrient deficiency in soil. Recently, the variety of fertilizers used in organic farming has increased and in addition to organic materials such as compost, humic and fulvic acids and leonardite, the production of fertilizers which contain different micro-organisms, enzymes and moss extracts have been started commercially. In studies using such fertilizers, it was reported that soil microbial activity was affected in different ways by seaweed extracts (Blunden, 1991), and leonardite (Tamer and Karaca, 2004; Karaca et al, 2006; Demirkıran et al., 2008). It was clearly carried out that addition to organic matter application, N, P, K fertilization was very important for pistachio (Weinbaum and Muraoka, 1989). However, studies on feeding of small pistachio trees (one-year-old) with organic and inorganic fertilizers are very limited in the scientific world (Aaliabadi, 2006). In a study, the effect of peat on the growth and nutrition of *Pistacia lentiscus* L. was tested. For growing media each compost was added at a rate of 40%, fresh pine bark at 20or 40% and peat at 20, 40 or 60%. In relation to plants growing in peat-based substrate (control), nutrition and P uptake was notably enhanced (Ostos et al., 2008). A greenhouse experiment was conducted to determine the effect of peat moss-shrimp wastes compost on barley (*Hordeum vulgare L.*) grown on a limed loamy sand soil. Results from this study indicate that peat moss-shrimp wastes compost could represent a potential means of renovating low fertility sand soils (Parent and Isfan, 1995).

In existing studies, some organic or inorganic materials were used separately as nutrition in the experiments, but they were not applied to small pistachio trees and seedlings simultaneously. Most of these studies conducted generally include pistachio trees. However, a suitable first growing stage and available nutrients are very important for a systematic plant growth. In this respect, the effects of different organic and inorganic materials on small pistachio trees was studied together and in utilizing, it is occasionally used to investigate their nutritional effects.

Therefore, the objective of this study is to investigate the effects of different organic and inorganic materials as nutrients on nutrient up taking ability of one-year old and 7 cm long pistachio (*P. vera* L.) seedlings by analyzing nutrient (mineral) contents of pistachio leaves. The experiment was conducted in a greenhouse by using six organic materials (gyttja, alsil, humic acid, sea moss, straw, and peat) and two chemical fertilizers (15-15-15, and 20-20-0) with different dosages.

MATERIALS AND METHODS

Materıals

General properties of organic materials

Alsil: It is obtained as a result of sieving and transformation prosesses of magma formed through transformations in billions of years in deep layers of earth. Alsil used in the treatment was supplied by Sinor Agricultural Company®, Istanbul (www.sinor.com.tr).

Sea moss: Submerged and decomposed formations of moss and other swamp plants. Moss is acidic (pH is 3.5 - 4.5), loose, very permeable, poor in terms of nutrients, and has very high water holding capacity. Plants and plant roots grow very well under these conditions (Parent and Isfan, 1995). Nutrient needs of mosses are fulfilled by absorption of airborne particles from wet and dry deposition. These ectohydric organisms have primitive tissues without cuticle or waxy layers, enabling water and elements to reach cell wall and membranes, or the cytoplasm by passive or active processes (Brown and Bates, 1990). The moss material used in the treatment was provided by Izotar Anonymous Company®, Istanbul.

Gyttja: Gyttja is a semi-formed lignite coal cover layer and is not used for fuel due to low calorie (Demirkıran et al., 2008). Gyttja is normally greenish in color, but may be brown or red. It bleaches on drying, usually to a grey color. In the wet state, gyttja has an elastic, rubbery consistency. It has a brittle rupture. It shrinks strongly on drying to form hard lumps with low density (Hartlén and Wolski, 1996). The pH of gyttja was 7.75, electrical conductivity (EC) was 0.68 dS m^{-1}, total organic C content was 25.5%, and $CaCO_3$ content was 32.5%. Total P content of gyttja was 17 mg kg^{-1}, and total N, 0.84%. The contents of humic and fulvic acids were 40.78 and 27.49%, respectively (Tamer and Karaca, 2004; Karaca et al., 2006). Gyttja samples used in the treatment were provided by Afşin-Elbistan Coal Mining Company® enterprise of Kahra-manmaraş province.

Humic acid: They are black or dark brown materials which are partially or fully decomposed plant or animal wastes. Main composition of soil organic materials is humus, which is the most important reasons of using humic acids in increasing soil fertility. The most important property of humic acids is their ability to combine insoluble metal ions, oxides, and hydroxides, and afterwards to release them to crops slowly and continuously when needed. The benefits of humic acids can be grouped as physical, chemical, and biological (Singh and Amberger, 1997; Çelik, 2003). Liquid humic acid used in the treatment was obtained from Izotar Anonymous Company®, Istanbul.

Straw: The residues of wheat used in the treatment grown in Kahramanmaraş region were grounded in a porcelain container and then used as straw.

Peat: Peat is an organic soil formed by plant growth when there is downfall of water level in lake beds and then death of plants when there is rise of water level in winter time, and through repetition of this natural event, transformations and accumulations of plant roots and stems take place for thousands of years. Most nurseries in the world have based, for many years, their growing media on peat. Peat is obtained from wetlands, which are being rapidly depleted, causing environmental concerns that have led to many individual countries to limit the extent of peat mining, and prices are

Table 1. Some physical and chemical properties of the study soil.

Soil property	Value
Texture	**Clay loam**
Saturation (%)	55
pH	7.6
Lime ($CaCO_3$,%)	5.6
Organic matter (%)	1.46
Total salt (%)	0.06
P_2O_5 (kg/ha)	4.6
K_2O (kg/ha)	655

increasing as a result. Research on peat alternatives is of great interest in the future (Ingelmo et al., 1998; Guerrero et al., 2002; Chong, 2005; Wilson et al., 2006). The combination of peat and compost in growing media is synergistic; peat often enhances aeration, water retention and compost or other additives improves the fertilizing capacity of a substrate. In addition, organic by-products and composts tend to have porosity and aeration properties comparable to those of bark or peat and, as such are ideal substitutes in propagating media (Chong, 2005). The peat used in the treatment was obtained from Malatya province of Turkey.

Methods

This study was conducted in a greenhouse using one-year old and about 7 cm length *P. vera* L. seedlings planted into 20 kg pots. Dosages of organic and inorganic materials were applied to these small trees and then the plants were irrigated regularly.

The experiment was conducted with an Inceptisol soil developed on alluvial sediments in the Aksu region of Kahramanmaraş. Twenty-kilogram portions of the soils, treated and thoroughly mixed with organic matter and inorganic fertilizers. Each treatments was replicated four times. The treatments which contained control, organic materials and inorganic fertilizers are given in the following:

Control

The control was carried out in 20 kg soil

Organic material

Alsil of 1/5, 2/5, and 3/5 as volume in 20 kg soil; sea moss of 1, 2, 4, and 8 g in 20 kg soil; gyttja of 10, 20, 40, and 80 g in 20 kg soil; humic acid of 1, 2, 5, and 10 ml in 20 kg soil; straw of 10, 20, 40, and 80 g in 20 kg soil; peat of 1/5, 2/5, and 3/5 as volume in 20 kg soil,

Chemical fertilizer

Chemical fertilizers used were 15-15-15 of 0.15, 0.3, 0.45, and 0.6 g in 20 kg soil as well as 20-20-0 of 0.75, 1.5, 2.25, and 3 g in 20 kg soil.

The application of nutrients was stopped when the small trees reached grafting thickness. After the application of different dosages of organic and inorganic nutrients, the nutrient contents of leaves for each treatment were determined. After one month of the experiment, the leaf samples were collected and analyzed for nutrients.

Analyses of macro and micro nutrients in the leaves

After taking 2 or 3 mature leaves from the small tree in each pot, the leaves were dried in oven at 60°C and then grinded. A sample of 0.5 g grinded leaf was exposed to burning by using the mixing of nitric and perchloric acid. After the completion of burning, the sample was diluted and then the instrumental analysis was conducted. While the elements of Ca, Mg, K, Na, Fe, Cu, Zn, and Mn were determined by atomic absorption spectrophotometer, AAS 3110 Perkin Elmer®, P was determined by spectrophotometer using the method of coloring with Barton solution (Jackson, 1973).

Analyses of soil

The soil sample was air-dried and passed through a 2-mm sieve before analysis. The soil pH was determined by glass electrode on saturated soil samples. Electrical conductivity of the soil was measured in saturation paste extract (Rhodes, 1996). Lime content of the soil was measured by the Scheibler calcimeter. Organic matter content of the soil was determined by the modified Walkley-Black wet oxidation procedure described by Nelson and Sommers (1996). The soil potassium content including exchangeable potassium was determined using the methods described in Knudsen et al. (1982). The soil texture was determined by the hydrometer method (Bouyoucous, 1951). The phosphorus content of the samples was determined by spectrophotometer, Jenway 6100, using the sodium bicarbonate method (Olsen and Sommers, 1982).

Statistical Analyses

Data tests were performed for evaluation of all experimental data such as measurements, counting's, and analysis results based on the randomized block design (SAS, 1989).

RESULTS

Soil properties and optimum values of nutrients in leaves

Some physical and chemical properties of soils used in the experiment is presented in Table 1. As shown in Table 1, experimental soil was slightly alkali in pH, clay loam in texture, mid level in lime content, low in organic matter, mid level in salinity, low in phosphorus content, and sufficient in potassium. The optimum values of nutrients in leaves for pistachio is tabulated in Table 2 according to Ashworth et al. (1985), Tekin et al. (1995) and Brown, (1995).

Effects of chemical fertilizers

Composed fertilizer as 15-15-15

The effect of fertilizer 15-15-15 on leaf nutrient contents of pistachio is tabulated in Table 3. The effect of fertilizer 15-15-15 on P, Na, and Cu contents of pistachio leaves was statistically significant, whereas, its effect on K, Ca, Mg, Zn, Mn, and Fe contents of pistachio leaves was statistically not significant. While fertilizer 15-15-15

Table 2. The optimum values of nutrients in leaves for pistachio grown in arid conditions according to some researchers.

Optimum values	P (%)	K (%)	Ca (%)	Mg (%)	Fe (ppm)	Zn (ppm)	Mn (ppm)	Cu (ppm)
Tekin et al. (1986)	0.06 - 0.1	0.8 - 1.2	2.2 - 3.7	0.5 - 0.9	108	17.5	35	48
Caruso et al. (2005)	0.35 - 0.4	0.8 - 1.9	0.2-0.6	0.7 - 4.7	33 - 130	17.2 - 37.3	13.3 - 28.6	7.3 - 31.1
Brown (1995)	0.14	1.00		0.60				
Ashworth et al. (1985)	0.24	0.7 - 0.9						

Table 3. The effect of fertilizer 15-15-15 on leaf nutrient contents of the pistachio seedlings leaves.

15-15-15 application	P**	K^{ns}	Ca^{ns}	Mg^{ns}	Na*	Zn^{ns}	Mn^{ns}	Cu*	Fe^{ns}
	ppm								
0.00	135.51b	12.75	604.14	245.74	12557.26a	16.29	24.82	21.79a	19.34
0.15	374.33a	16.30	686.43	187.65	658.21b	6.11	15.33	6.00b	31.35
0.30	423.98a	15.20	644.13	199.07	511.94b	4.25	13.87	8.20b	6.40
0.45	423.98a	14.10	1028.34	253.23	628.96b	4.90	30.78	3.20b	13.37
0.60	413.24a	12.75	671.07	217.16	504.63b	3.48	24.57	5.80b	23.69

^{ns}Not significant (LSD test), *significant at p = 0.05 level (LSD test), **significant at p = 0.01 level (LSD test).

Table 4. The effect of fertilizer 20-20-0 on leaf nutrient contents of the pistachio seedlings leaves.

20-20-0 application	P^{ns}	K^{ns}	Ca^{ns}	Mg^{ns}	Na*	Zn^{ns}	Mn^{ns}	Cu*	Fe^{ns}
	ppm								
0.00	135.51	12.75	604.14	245.74	12557.26a	16.29	24.82	21.79a	19.34
0.75	414.59	13.73	571.98	190.28	387.62b	6.01	20.19	6.40b	28.11
1.50	300.54	16.55	471.72	228.50	380.30b	4.08	7.77	4.80b	21.90
2.25	222.72	16.18	482.73	221.72	475.38b	4.46	21.65	4.20b	25.18
3.00	328.05	14.71	522.72	202.51	497.32b	3.73	17.27	3.20b	15.35

^{ns} Not significant (LSD test), *significant at p = 0.05 level (LSD test).

application increased P content of pistachio leaves to a significant level, its application decreased Na and Cu contents of the leaves at significant level. Durzan (1995) reported that zinc deficiency of pistachio trees was characterized visually as 'little leaf', analytically by low levels of zinc, and internally by increase in N, P and total soluble N.

Composed fertilizer as 20-20-0

The effect of fertilizer 20-20-0 on leaf nutrient contents of pistachio is tabulated in Table 4. The effect of fertilizer 20-20-0 on Na and Cu contents of pistachio leaves was statistically significant, whereas, its effect on P, K, Ca, Mg, Zn, Mn, and Fe contents of pistachio leaves was statistically not significant. The fertilizer 20-20-0 application decreased both Na and Cu contents of pistachio leaves to a significant level. Cu content decreased with the application of 20-20-0 fertilizer. Durzan (1995) also reported that zinc deficiency of pistachio trees was characterized analytically by low levels of zinc, and internally by increases in N, P and total soluble N.

Effect of organic materials

Alsil

The effect of alsil application on leaf nutrient contents of pistachio is tabulated in Table 5. Alsil application had no statistically significant effect on nutrient contents of pistachio leaves except Cu content in which Cu content decreased at significant level. Durzan (1995) also reported that decrease of zinc content of pistachio trees was characterized analytically by low levels of zinc, and internally by increases in N and P. Furthermore, alsil materials which have been included in magmatic characteristic as mentioned above increased calcium content of pistachio leaves. These properties are important for trees which have calcium deficiencies.

Sea moss

The effect of sea moss application on leaf nutrient

Table 5. The effect of alsil on leaf nutrient contents of the pistachio seedlings leaves.

Alsil application	P^{ns}	K^{ns}	Ca^{ns}	Mg^{ns}	Na^{ns}	Zn^{ns}	Mn^{ns}	Cu*	Fe^{ns}
	ppm								
0	135.51	12.75	604.14	245.74	12557.26	16.29	24.82	21.79a	19.34
1	251.57	10.42	1052.68	266.50	650.90	4.51	31.02	4.00b	12.10
2	48.30	12.26	967.78	267.50	585.08	5.51	24.94	2.40b	21.50
3	124.78	12.26	1039.35	253.86	643.59	5.51	28.59	2.40b	24.75

^{ns}Not significant (LSD test), *significant at p = 0.05 level (LSD test).

Table 6. The effect of sea moss application on leaf nutrient contents of the pistachio seedlings leaves.

Sea moss application	P^{ns}	K^{ns}	Ca^{ns}	Mg*	Na^{ns}	Zn^{ns}	Mn*	Cu^{ns}	Fe^{ns}
	ppm								
0	135.51	12.75	604.14	245.74a	12557.26	16.29	24.82b	21.79	19.34
1	714.46	13.48	658.03	113.21ab	1016.58	2.68	223.71a	281.92	37.66
2	1233.69	14.22	673.97	46.19b	1908.82	11.65	182.71ab	235.74	20.13
4	1007.62	10.79	884.91	44.78b	2881.52	13.83	13.14b	254.33	16.62
8	72.45	10.91	361.04	73.51b	7284.24	23.89	21.53b	366.30	23.75

^{ns}Not significant (LSD test), *significant at p = 0.05 level (LSD test).

Table 7. The effect of gyttja application on leaf nutrient contents of the pistachio seedlings leaves.

Gyttja application	P^{ns}	K^{ns}	Ca^{ns}	Mg**	Na^{ns}	Zn^{ns}	Mn^{ns}	Cu^{ns}	Fe^{ns}
	ppm								
0	135.51	12.75	604.14	245.74a	12557.26	16.29	24.82	21.79	19.34
10	292.49	9.93	143.43	49.97b	314.48	5.95	48.66	255.93	7.68
20	268.34	8.95	339.59	46.63b	3583.61	7.98	39.29	205.34	17.89
40	216.69	7.97	390.59	58.24b	5046.31	5.66	50.00	214.14	13.56
80	275.72	8.46	364.51	50.30b	4073.61	5.20	44.16	38.99	15.54

^{ns} Not significant (LSD test), **significant at p = 0.01 level (LSD test).

contents of pistachio is tabulated in Table 6. The effect of sea moss application on Mg and Mn contents of pistachio leaves was statistically significant, but its effect on P, K, Ca, Na, Zn, Cu, and Fe contents of pistachio leaves was statistically not significant. Sea moss treatments enhanced phosphorus content of pistachio leaves due to sea materials having significant phosphorus element. Through this, organic material was treated to the plants and phosphorus contents of pistachio leaves were raised as mentioned by Ben Mimoun et al. (2005) and Tekin et al., (1986). Phosphorus content of leaves were not enhanced to the optimum level of phosphorus by other organic and inorganic nutrient treated. While sea moss application decreased Mg content of pistachio leaves to a significant level, its application first increased and then decreased Mn content of the leaves to a significant level. The application of sea moss which is rich in nutrients (Blunden, 1991), increased Cu content of leaves and also the applications up to certain level increased P and Mn contents. In the higher sea moss application, it should be carefully applied for potential salinity problem in soil.

Gyttja

The effect of gyttja application on leaf nutrient contents of pistachio is tabulated in Table 7. Gyttja application had no statistically significant effect on nutrient contents of pistachio leaves except Mg content in which Mg content decreased at significant level. Gyttja was obtained from the Afsin–Elbistan Lignite deposits (Kahramanmaraş, Eastern Turkey). Since EC and $CaCO_3$ of gyttja are high (Tamer and Karaca, 2004; Karaca et al., 2006; Demirkıran et al., 2008), K, Ca, Mg, Zn (Durzan, 1995), and Fe contents of pistachio leaves decreased. Due to high phosphorus content of gyttja, P contents of pistachio leaves increased. As in the higher sea moss application,

Table 8. The effect of humic acid application on leaf nutrient contents of the pistachio seedlings leaves.

Humic acid application	P***	K^{ns}	Ca^{ns}	Mg^{ns}	Na*	Zn^{ns}	Mn^{ns}	Cu*	Fe^{ns}
	ppm								
0	135.51c	12.75	604.14	245.74	12557.26a	16.29	24.82	21.79a	19.34
10	464.90a	9.19	297.87	118.96	299.85b	4.17	14.96	4.20b	28.25
20	452.15a	11.28	734.82	215.82	504.63b	6.77	27.49	7.20b	21.27
40	360.25ab	8.58	725.84	193.65	511.94b	6.03	19.83	6.00b	27.78
80	251.57bc	8.09	496.06	149.95	358.36b	5.50	14.60	5.00b	29.48

^{ns}Not significant (LSD test), *significant at p = 0.05 level (LSD test), **significant at p = 0.01 level (LSD test).

Table 9. The effect of straw application on leaf nutrient contents of the pistachio seedlings leaves.

Straw application	P**	K^{ns}	Ca^{ns}	Mg^{ns}	Na^{ns}	Zn^{ns}	Mn^{ns}	Cu^{ns}	Fe^{ns}
	ppm								
0	135.51b	12.75	604.14	245.74	12557.26	16.29	24.82	21.79	19.34
10	271.69a	9.07	278.46	134.90	277.91	5.12	15.81	4.40	14.98
20	303.90a	10.79	263.39	261.64	221.89	3.13	18.49	5.80	14.78
40	318.65a	11.03	1218.71	221.86	277.91	4.34	23.96	5.20	30.53
80	370.31a	10.30	285.12	178.68	263.29	2.96	14.60	7.20	45.20

^{ns}Not significant (LSD test), **significant at p = 0.01 level (LSD test).

Table 10. The effect of peat application on leaf nutrient contents of the pistachio seedlings leaves.

Peat application	P**	K^{ns}	Ca^{ns}	Mg^{ns}	Na^{ns}	Zn^{ns}	Mn^{ns}	Cu^{ns}	Fe^{ns}
	ppm								
0	135.51b	12.75	604.14	245.74	12557.26	16.29	24.82	21.79	19.34
1	392.45a	10.42	911.86	251.59	570.45	21.77	101.33	293.32	23.91
2	348.84a	11.89	444.19	182.72	497.32	27.70	197.92	478.07	30.44
3	392.45a	12.63	711.93	216.93	519.26	27.23	232.59	160.76	37.19

^{ns}Not significant (LSD test), **significant at p = 0.01 level (LSD test).

gyttja should be carefully used as organic material due to salinity problem, potentially.

Humic acid

The effect of humic acid application on leaf nutrient contents of pistachio is tabulated in Table 8. The effect of humic acid application on P, Na, and Cu contents of pistachio leaves was statistically significant, whereas, its effect on K, Ca, Mg, Zn, Mn, and Fe contents of pistachio leaves was statistically not significant. While humic acid application increased P content of pistachio leaves at significant level, its application decreased K, Ca, Mg, Na, Zn (Durzan, 1995) and Cu, especially Na and Cu contents of the leaves at significant level. Humic acid had the same effect on nutrient contents of pistachio leaves as fertilizer 15-15-15. In recent studies, the solubility of phosphorus in soil increased due to humic and fulvic acids (Singh and Amberger, 1997). In this study, humic acid increased phosphorus content of pistachio leaves at significant level parallel to the fact that the solved phosphorus is better used by plants.

Straw

The effect of straw application on leaf nutrient contents of pistachio is tabulated in Table 9. Straw application had no statistically significant effect on nutrient contents of pistachio leaves except P content which increased at significant level. While straw application increased P content of pistachio leaves, its application decreased K, Ca, Na, Zn (Durzan, 1995) and Cu contents.

Peat

The effect of peat application on leaf nutrient contents of pistachio is tabulated in Table 10. Peat application had no statistically significant effect on nutrient contents of pistachio leaves except P content which increased at

significant level. Many researchers (Ostos et al., 2008; Parent and Isfan, 1995; Chong, 2005) reported that peat facilitate and increase the uptake of nutrients by many plants.

DISCUSSION

As reported by several researchers (Tekin et al., 1995; Tekin and Güzel, 1992), soils of pistachio trees significantly lack many plant nutrients and had generally low level of organic matter content in Turkey. Using inorganic fertilizers for nutrient supplement of the pistachio trees (Weinbaum et al., 1994; Brown, 1995; Durzan, 1995; Barone, 1997; Barone et al., 1998; Qiupeng et al., 1998; Zeng et al., 1998; Mimoun et al., 2005; Tavallali and Rahemi, 2007; Malakouti and Mozaffari, 2009; Mozaffari et al., 2009; Hosseinifard et al., 2010) and organic materials with soil improves soil structure and supply plant with nutrients (Weinbaum and Muraoka, 1989; Blunden, 1991; Parent and Isfan, 1995; Tamer and Karaca, 2004; Karaca et al., 2006; Aaliabadi, 2006; Ostos et al., 2008; Demirkıran et al., 2008).

This study showed that chemical fertilizers (15-15-15 and 20-20-0) negatively affected micro nutrients especially Zn content and statistically, Cu content of plant leaves. This situation can be assumed for those fertilizers that have macro nutrients, especially phosphorus whose interaction relationship between phosphorus and zinc nutrient is known (Durzan, 1995). These fertilizers increased phosphorus contents of pistachio leaves with low levels. This is shown in Table 3 (Ashworth et al., 1985, Tekin et al., 1995; Brown, 1995) and were reported by Aydeniz (1990) and Ak and Fidan (2009).

In addition, it has been determined that alsil, humic acid and straw in these organic materials decreased zinc and cupper content of pistachio leaves, and alsil and humic acid decreased cupper content in the plant leaves, statistically. This result indicated that these organic materials have been naturally macro nutrients.

In this study, moss had positive effect on Mn uptake by pistachio trees, whereas peat, straw, and humic acid positively affected P uptake. The possible reasons for these positive effects may be direct and indirect effects of organic materials on soils. Besides, inorganic fertilizers increased P content of pistachio leaves, but this increase caused decrease of Cu content. High level of P content may be the reason for the low uptake of some micro elements such as Cu. Although application of organic and inorganic materials generally increases P uptake, organic materials especially peat, gyttja and sea moss increased nutrient contents of pistachio leaves. This important knowledge is applied in areas where pistachio lacks nutrients.

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Full Length Research Paper

Evaluation of chemical and fermentation parameters during the preparation of wine from berry (*Maesobotyra standii*)

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A milky red wine was produced from locally sourced berry (*Maesobotyra standii*) in Abraka, Delta State, Nigeria using innate wild yeast (*Saccharomyces cerevisiae)* augmented with baker's yeast activated with 2, 5 and 10% (w/v) sugar solutions in activated yeast: fruit extract ratios of 1:2 and 1:3. It was observed that there was an inverse proportional relationship between percentage (%) titratable acidity and pH as well as for specific gravity (SG) and percentage (%) alcohol for 48 h after which both SG and % alcohol increased. Diauxic growth and malo-lactic fermentation were observed. Total aerobic counts followed same trend as for % alcohol. pH, % titratable acidity, specific gravity, % alcohol and total aerobic counts at 72 h fermentation ranged from 4.85 - 5.85, 0.955 - 1.152, 0.956 - 1.141, 0.54 - 2.62 and 0.95 - 185 x 10^3 cfu/ml, respectively. The baker's yeast activated with 5% (w/v) granulated sugar was most suitable for home or commercial berry wine production which gave wine with lower alcohol-content (1.84%, v/v alcohol) using 1:2 activated baker's yeast : fruit extract ratio; it gave wine with slightly higher alcohol-content (2.62%, v/v alcohol) with 1:3 baker's yeast : fruit extract ratio. Thus portable and/or commercial wine can be produced from berry (*M. standii*) with baker's yeast activated with 5% (w/v) granulated sugar.

Key words: Berry wine, sugar, baker's yeast, malo-lactic, fermentation.

INTRODUCTION

Wine making is gardening of a sort with selected yeasts being the seeds. They need nutrients in the form of sugar and all the elements associated with life. Selection of a good seed ensures a good wine (Anon, 2008b). Wild yeasts are found in almost all fruits but generally produce wine inferior in flavor and of lower alcohol content than wines produced with selected strains of yeast (Anon, 2008a).

Wine can be made from grapes, fruits and berries (Armstrong, 2004; Anon, 2007). Any small, fleshy fruit is popularly called a berry, especially if it is edible (Encyclopaedia Britannica, 2009). However, irrespective of the starting materials, there must be fermentation to produce alcohol and if the alcohol-content is relatively low, the result is wine. Various types of wines abound depending on the clarity and alcohol-content (Anon, 2008a, b, c, d). There can be a secondary fermentation, malo-lactic, when malic acid is broken down to lactic acid and carbon dioxide which imparts additional flavor, often 'buttery' to the wine (Todd, 1999; Anon, 2008d).

Berries, which are grape-like fruit clusters, belong to the family Euphorbiaceae and Genus Maesobotyra. They grow wildly throughout the seasons with white flavors but produce fruits between April and June, which is the onset of the rainy season. On maturation, the fruits are bright red containing fleshy edible pulp with red seed. The fruit is ovoid or ellipsoidal in shape with pointed styles about one centimeter long and broad. The genus is found to extend from South-western Nigeria to Zaire (Keay, 1989). It is known by different names in different tribes; 'oshushu' (Urhobo) and 'osunsun' (Ika).

Various berries have been used in wine making. With few exceptions, the more the fruits used in wine making, the fruitier the taste (Anon, 2008a). The amount of sugar

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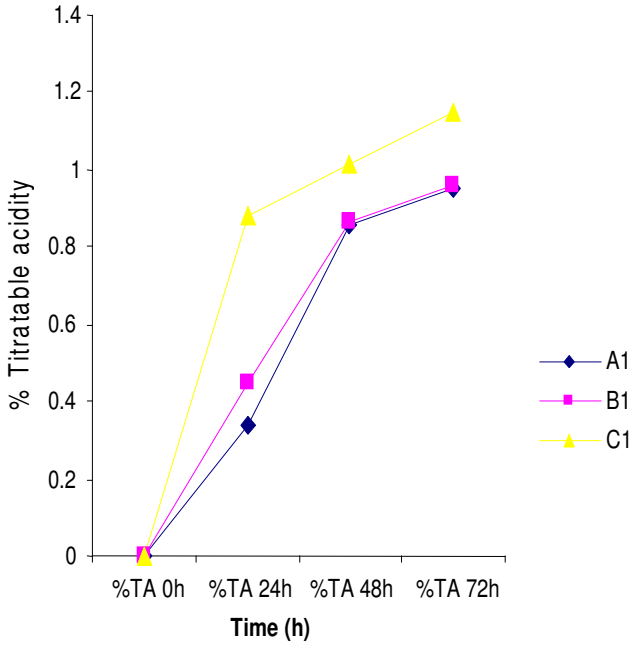

Figure 1a. Changes in % titratable acidity with fermentation of 2, 5 and 10% (w/v) sugar solutions (A1, B1 and C1, respectively), mixed with the juice at a ratio of 1:2.

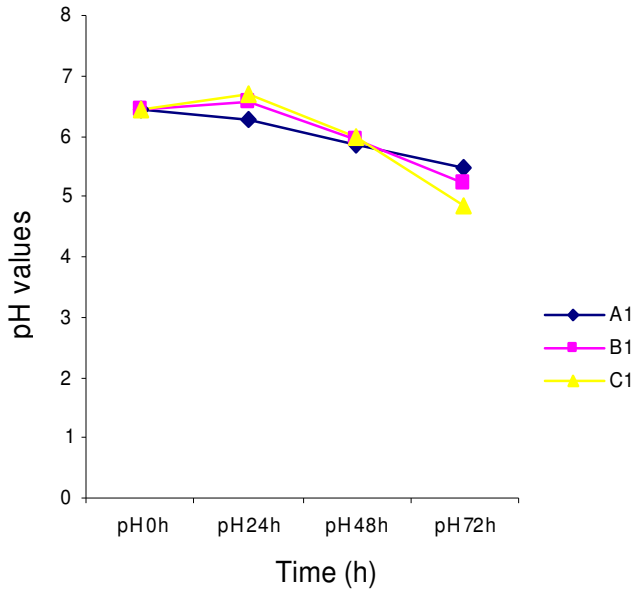

Figure 1b. Changes in pH with fermentation of 2, 5 and 10% (w/v) sugar solutions (A1, B1 and C1, respectively), mixed with the juice at a ratio of 1:2.

added depends on the amount naturally present. Tartness, usually caused by acid, could be produced by tannin, pectin or simply a natural flavor. These are usually removed by precipitation during wine maturation. They often affect the clarity of the wine (Armstrong, 2004).

The berry contains soluble carbohydrate (glucose and fructose) which enables it serve as food, energizer and can be used for the production of wine (Keay, 1989). It contains inherent yeasts of the genus *Saccharomyces* just as have been reported for various wild plants (Dias et al., 2006). The current study was aimed at the use of the berry in home and/or commercial wine production.

MATERIALS AND METHODS

Collection of berries, sugar and yeast

The berries, granulated sugar and baker's yeast (*Saccharomyces cerevisiae*) (Levure Instantance, 500 g net wt., Made in China) were purchased from Abraka main market, Abraka, Delta State, Nigeria. The berries were washed with sterile distilled water, crushed to extract the juice with a pestle in a 1 l plastic bowl. The pestle and plastic bowl were previously washed with detergent ('Omo') and sterilized by washing in 95% ethanol, placed in an inverted position and allowed to dry. The juice was stored at 4°C until required.

Preparation of the berry wine

Using three sterile 250 ml flasks, 2, 5 and 10% (w/v) sugar solutions, denoted as A, B and C respectively, were prepared to activate the baker's yeast. The activated yeast was mixed with the juice at ratios of 1:2 and 1:3 to give A1, B1, C1 and A2, B2, C2, respectively. Each flask was mixed properly by hand agitation, covered properly with a stopper that has a tube covered with cotton wool to release the carbon-dioxide that would be produced during the fermentation. pH, retention factor (R_f), specific gravity (SG), % titratable acidity (%TA) and total aerobic mesophilic counts (TAC) and % alcohol (w/v) were determined at 1, 24, 48 and 72 h for the fermenting broths.

Determination of pH, R_f,, % alcohol, specific gravity, % titratable acidity and TAC

pH was determined using a Mettler pH meter that was standardized in accordance with the manufacturer's instructions. The R_f values, % alcohol, specific gravity and % titratable acidity were carried out using the methods reported by Ogunkoye and Olubayo (1977). The TAC were carried out using the pour plate method reported by Cowan and Steel (2004).

RESULTS

The results of berry wine prepared from baker's yeast activated with 2, 5 and 10% (w/v) sugar solutions in activated yeast: fruit extract ratio of 1:2 is presented in Figure 1 while wine prepared from activated yeast: fruit extract ratio of 1:3 is presented in Figure 2. It was observed that there was an inverse proportional relationship between % titratable acidity and pH (Figures 1a, b, 2a and b) as well as for specific gravity and % alcohol for 48 h after which both SG and % alcohol increased (Figures 1c, d, 2c and d). The total aerobic counts observed the same trend with % alcohol (Figures 1e and 2e). It was observed that the 5% (w/v) sugar activated yeast produced wines with higher % alcohol-contents. The wine produced using 1:3 activated yeast: fruit extract ratio possessed the highest% alcohol-content of 2.62.

The results of the wine prepared from activated yeast: fruit extract ratios of 1:2 and 1:3 are presented in Figures 3 - 5 for 2, 5

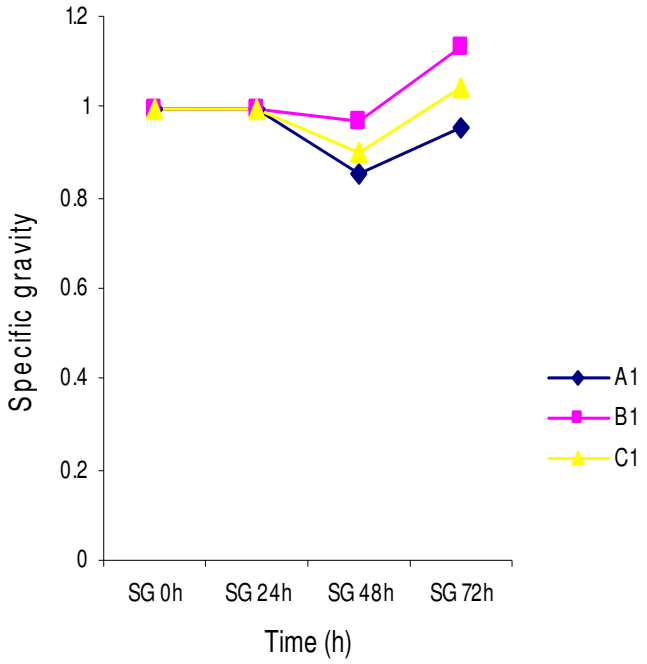

Figure 1c. Changes in specific gravity with fermentation of 2, 5 and 10% (w/v) sugar solutions (A1, B1 and C1, respectively), mixed with the juice at a ratio of 1:2.

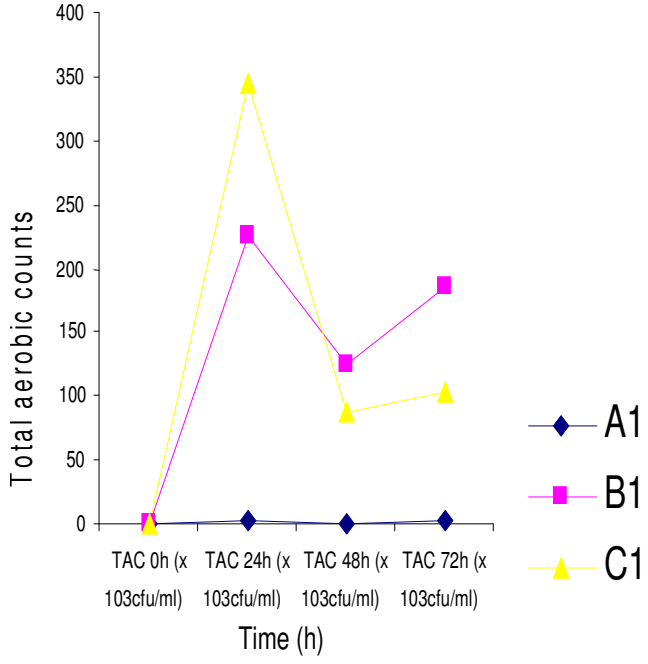

Figure 1e. Changes in total aerobic counts with fermentation of 2, 5 and 10% (w/v) sugar solutions (A1, B1 and C1, respectively), mixed with the juice at a ratio of 1:2.

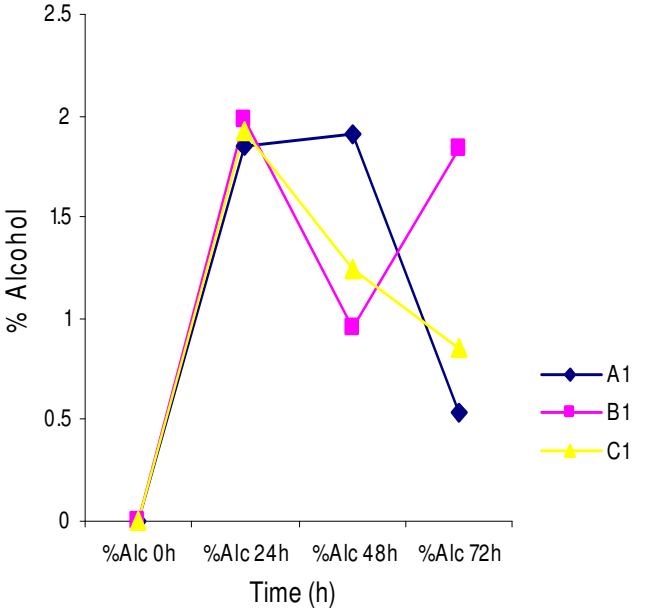

Figure 1d. Changes in % alcohol with fermentation of 2, 5 and 10% (w/v) sugar solutions (A1, B1 and C1, respectively), mixed with the juice at a ratio of 1:2.

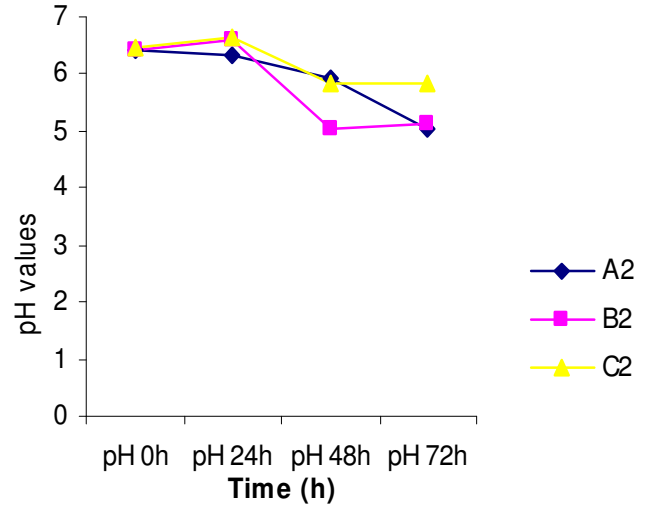

Figure 2a. Changes in % titratable acidity with fermentation of 2, 5 and 10% (w/v) sugar solutions (A2, B2 and C2, respectively), mixed with the juice at a ratio of 1:3.

and 10% (w/v) sugar respectively. It was observed that the wines produced from the 1:3 activated yeast: fruit extract ratios possessed higher values with the wine produced from 5% (w/v) activated yeast possessing highest value of 2.62. Diauxic growth was observed to take place after 48 h of fermentation as both alcohol and total aerobic counts increased (Figures 1c, d, e, 2c, d, e, 3b, c, d, 4c, d, e, 5c, d and e). A malo-lactic fermentation was observed to take place after 48 h as shown in the R_f values in Table 1.

DISCUSSION

The inverse relationship observed between pH and %

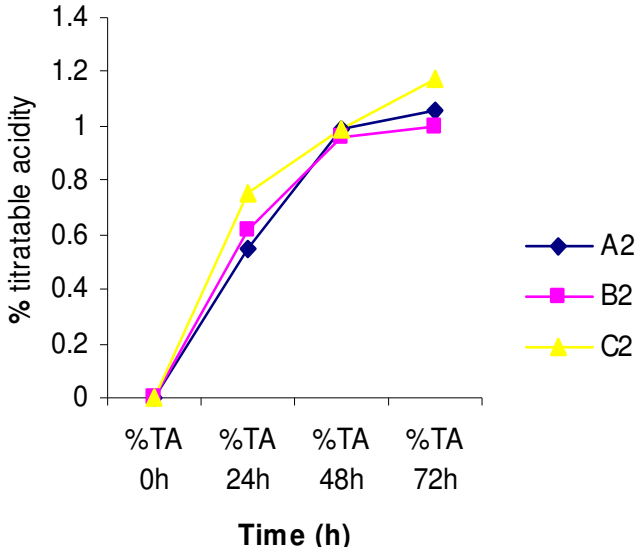

Figure 2b. Changes in pH with fermentation of 2, 5 and 10% (w/v) sugar solutions (A2, B2 and C2, respectively), mixed with the juice at a ratio of 1:3.

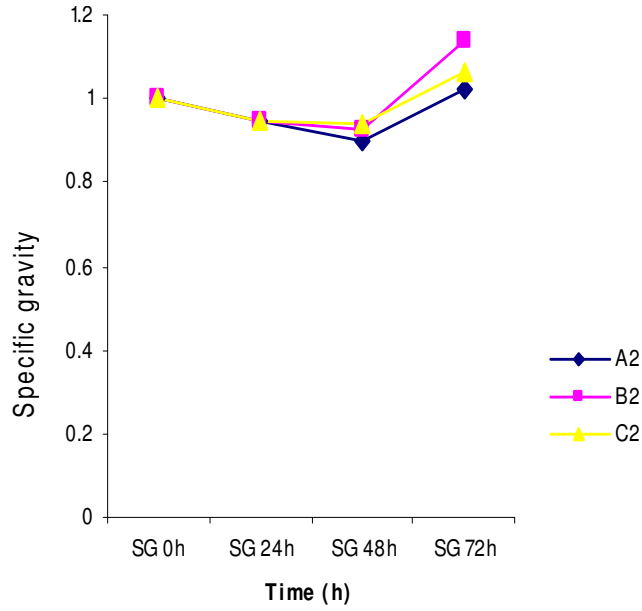

Figure 2c. Changes in specific gravity with fermentation of 2, 5 and 10% (w/v) sugar solutions (A2, B2 and C2, respectively), mixed with the juice at a ratio of 1:3.

titratable acidity and specific gravity and % alcohol in Figures 1 and 2 conforms to the reports of previous studies (Armstrong, 2004; Dias et al., 2006).

The increase in total aerobic counts, % alcohol and % titratable acidity after a decline phase observed in all the Figures indicate a diauxic growth. Diauxic growth observed could be due to the presence of sucrose in the fruit. Sucrose, a disaccharide, is made up of a molecule

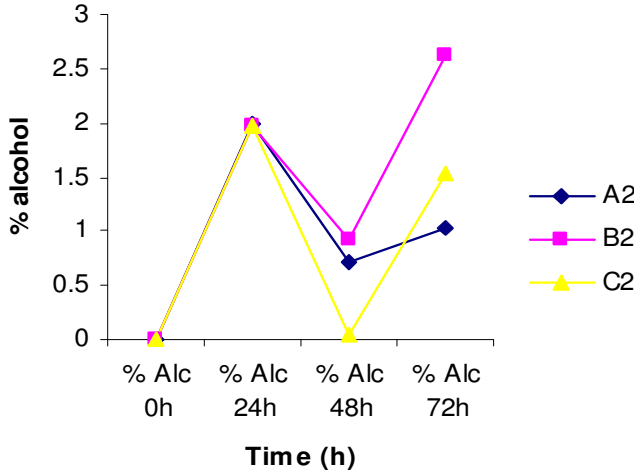

Figure 2d. Changes in % alcohol with fermentation of 2, 5 and 10% (w/v) sugar solutions (A2, B2 and C2, respectively), mixed with the juice at a ratio of 1:3.

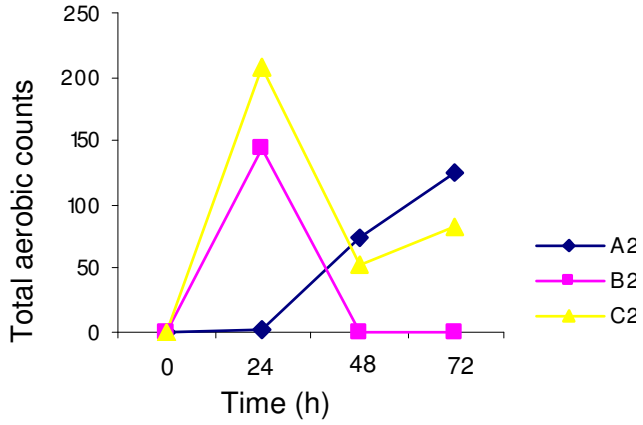

Figure 2e. Changes in total aerobic counts with fermentation of 2, 5 and 10% (w/v) sugar solutions (A2, B2 and C2, respectively), mixed with the juice at a ratio of 1:3.

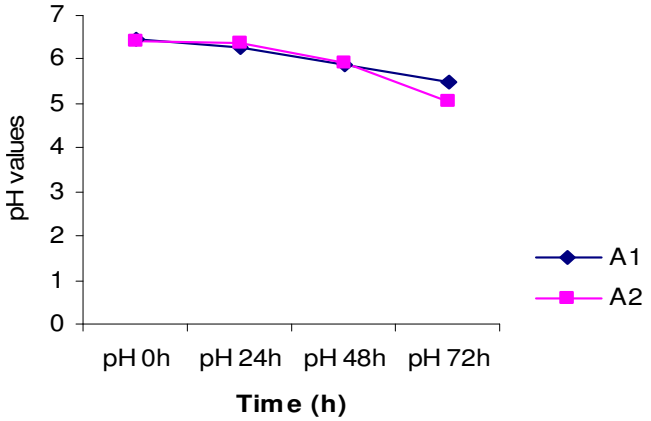

Figure 3a. Changes in pH with fermentation of 2% (w/v) sugar solutions, mixed with the juice at a ratio of 1:2 and 1:3 (A1 and A2, respectively).

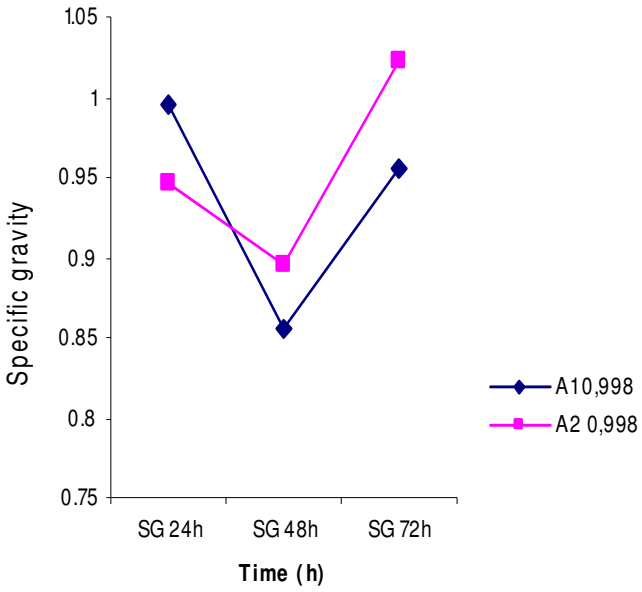

Figure 3b. Changes in specific gravity with fermentation of 2% (w/v) sugar solutions, mixed with the juice at a ratio of 1:2 and 1:3 (A1 and A2, respectively).

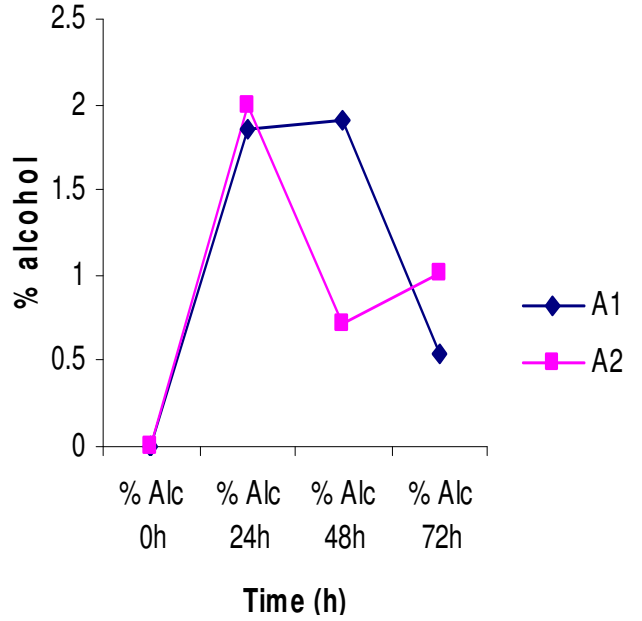

Figure 3c. Changes in % alcohol with fermentation of 2% (w/v) sugar solutions, mixed with the juice at a ratio of 1:2 and 1:3 (A1 and A2, respectively).

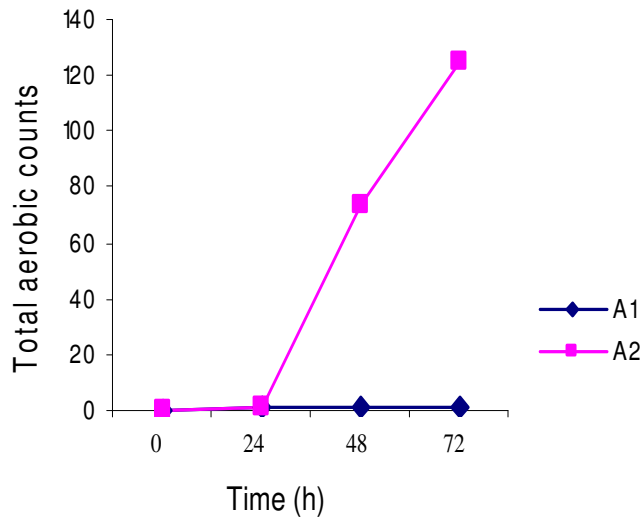

Figure 3d. Changes in total aerobic counts with fermentation of 2% (w/v) sugar solutions, mixed with the juice at a ratio of 1:2 and 1:3 (A1 and A2, respectively).

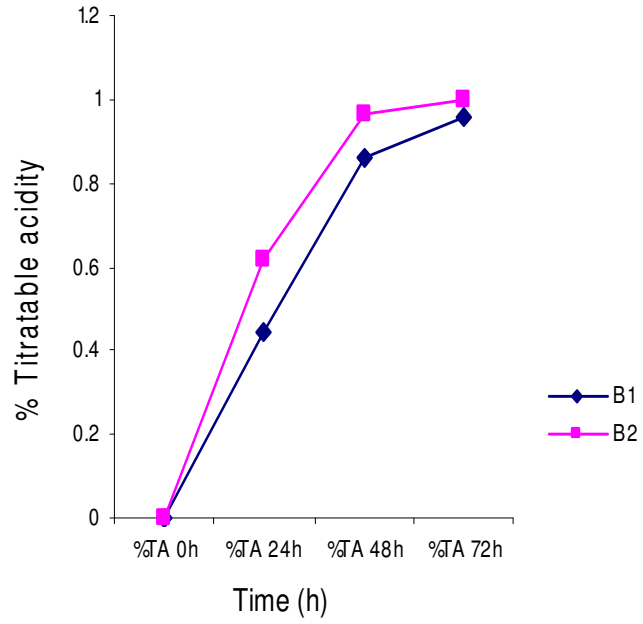

Figure 4a. Changes in % titratable acidity with fermentation of 5% (w/v) sugar solutions, mixed with the juice at a ratio of 1:2 and 1:3 (B1 and B2, respectively).

of glucose and a molecule of fructose. In the presence of two sources of metabolizable nutrients, an organism usually utilizes the most readily available source to exhaustion and thereafter utilizes the second thereby resulting in a log phase following a death phase (Precott et al., 2008). This is in agreement with reports of previous studies (Dias et al., 2006).

The higher values observed for wines produced from 1:3 ratio of activated yeast: fruit extract indicate the ready availability of the required growth nutrients at this dilution. This conforms to reports of previous studies which support the determination of optimal conditions for microbial actions in food production processes (Anon, 2008a, b, c, d; Precott et al., 2008).

The observed R_f values indicate the presence of a malo-lactic fermentation. This reportedly imparts a

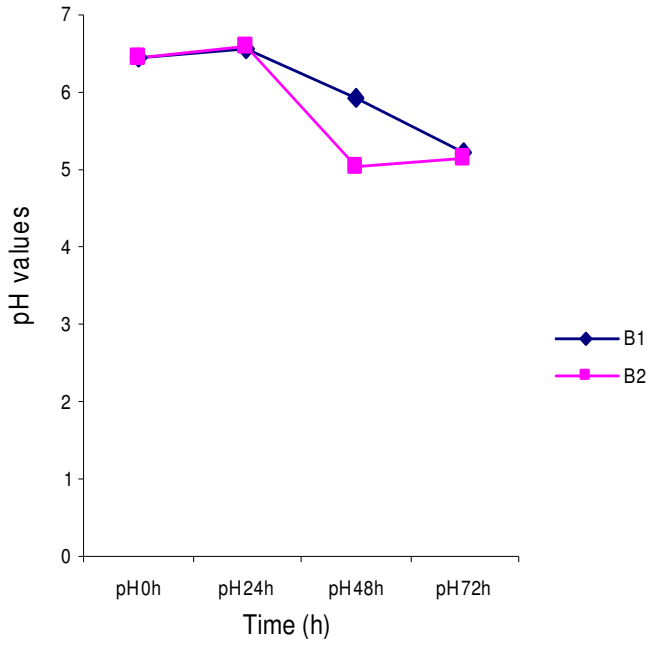

Figure 4b. Changes in pH with fermentation of 5% (w/v) sugar solutions, mixed with the juice at a ratio of 1:2 and 1:3 (B1 and B2, respectively).

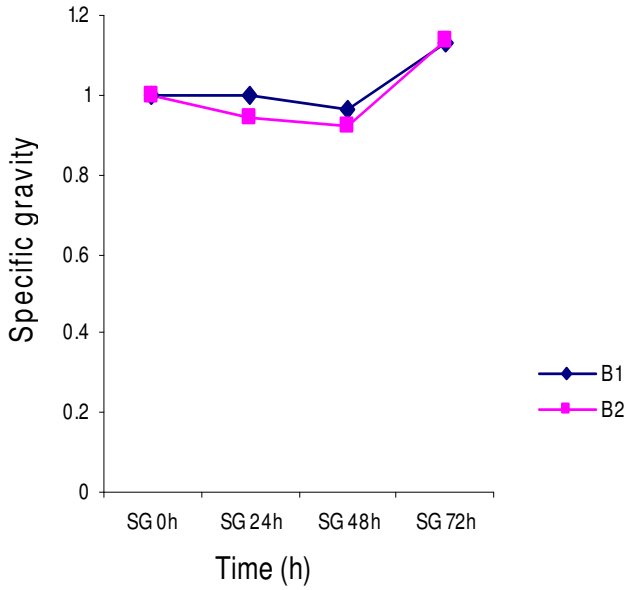

Figure 4c. Changes in specific gravity with fermentation of 5% (w/v) sugar solutions, mixed with the juice at a ratio of 1:2 and 1:3 (B1 and B2, respectively).

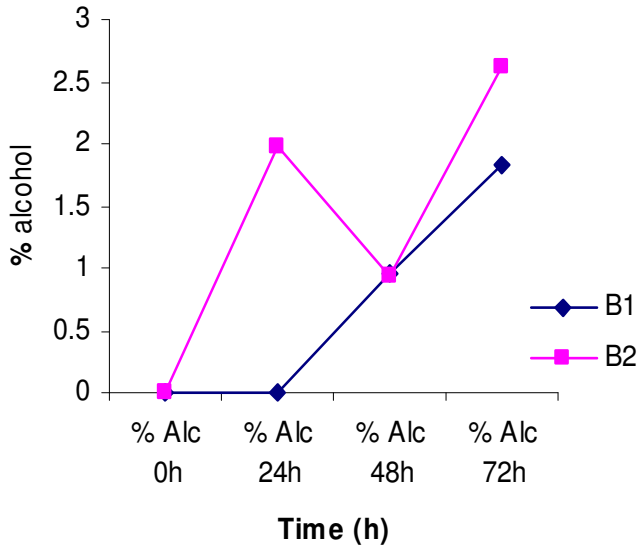

Figure 4d. Changes in % alcohol with fermentation of 5% (w/v) sugar solutions, mixed with the juice at a ratio of 1:2 and 1:3 (B1 and B2, respectively).

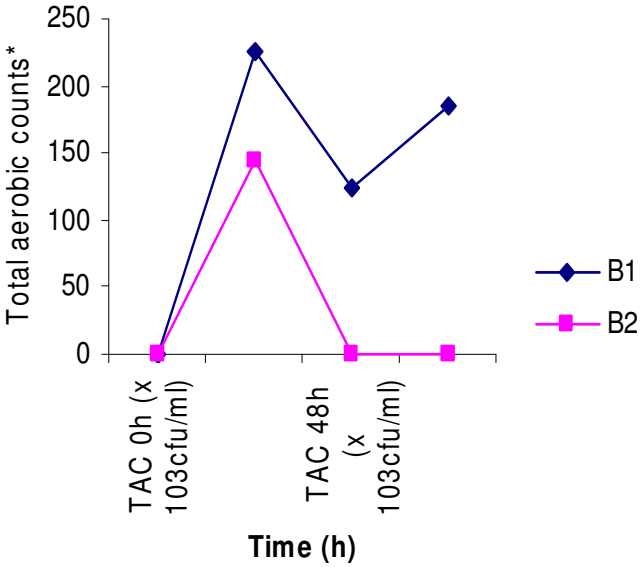

Figure 4e. Changes in total aerobic counts with fermentation of 5% (w/v) sugar solutions, mixed with the juice at a ratio of 1:2 and 1:3 (B1 and B2, respectively).

desirable flavor often 'buttery' to the wine during maturation. This is in agreement with reports of previous studies (Armstrong, 2004; Dias et al., 2006; Anon, 2008b; Prescott et al., 2008). The alcoholic fragrance was stronger in B2 than B1 presumably due to the higher alcoholic content. This is in agreement with the report of Anon (2008b).

In conclusion, a red, milky slightly alcoholic wine was produced from locally sourced berry (*Maesobotyra standii*) augmenting with Baker's yeast activated with various concentrations of granulated sugar. The baker's yeast activated with 5% (w/v) granulated sugar is most suitable for home or commercial berry wine production. A wine with lower alcohol-content (1.84%, v/v alcohol) could be made using 1:2 activated baker's yeast: fruit extract ratio while wine with slightly higher alcoholic

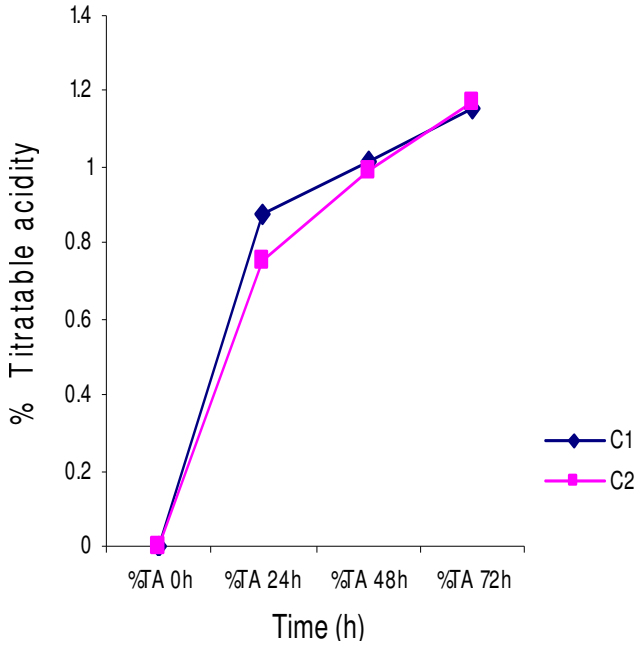

Figure 5a. Changes in % titratable acidity with fermentation of 10% (w/v) sugar solutions, mixed with the juice at a ratio of 1:2 and 1:3 (C1 and C2, respectively).

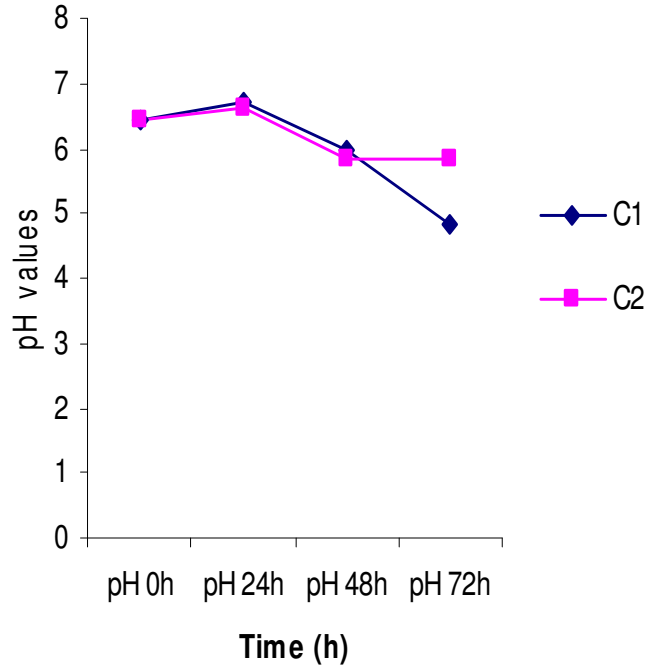

Figure 5b. Changes in pH with fermentation of 10% (w/v) sugar solutions, mixed with the juice at a ratio of 1:2 and 1:3 (C1 and C2, respectively).

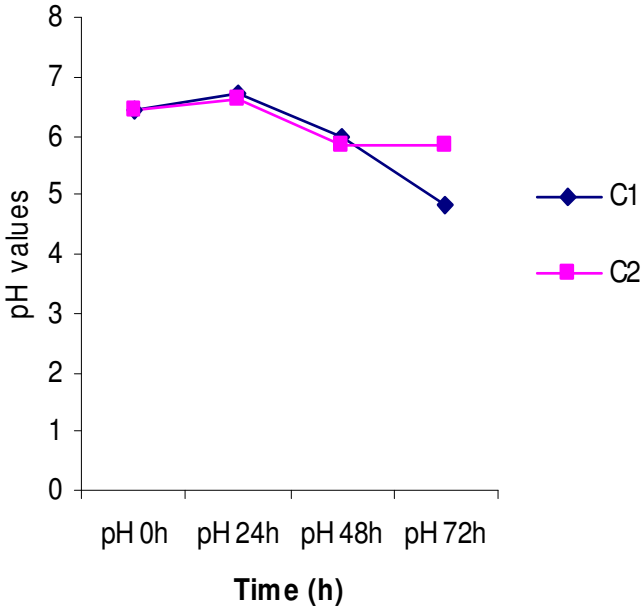

Figure 5b. Changes in pH with fermentation of 10% (w/v) sugar solutions, mixed with the juice at a ratio of 1:2 and 1:3 (C1 and C2, respectively).

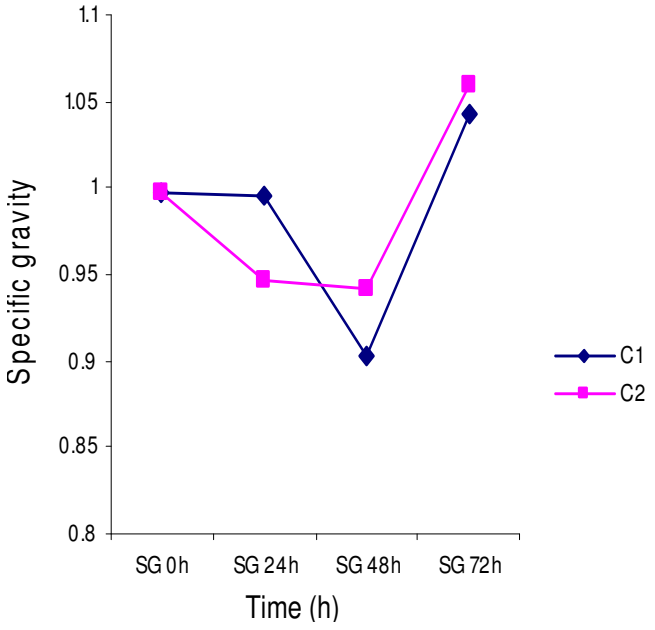

Figure 5c. Changes in specific gravity with fermentation of 10% (w/v) sugar solutions, mixed with the juice at a ratio of 1:2 and 1:3 (C1 and C2, respectively).

content (2.62% v/v alcohol) could be produced with 1:3 baker's yeast: fruit extract ratio. A diauxic growth was observed apparently due to presence of two growth nutrients. A malo-lactic fermentation was also observed which reportedly imparts a desirable 'buttery' flavor on the wine during maturation. Thus portable and/or commercial wine can be produced from berry (*M. standii*) with baker's yeast activated with 5% (w/v) granulated sugar.

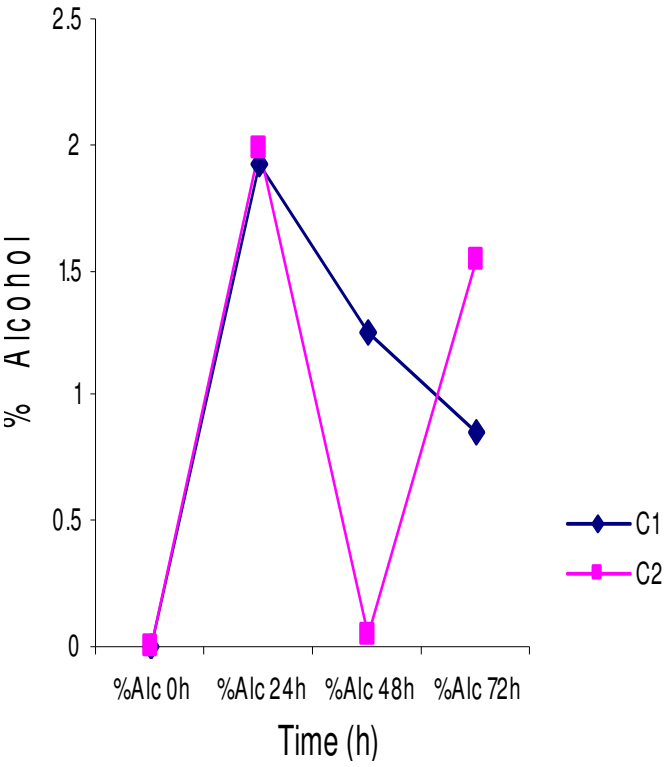

Figure 5d. Changes in % alcohol with fermentation of 10% (w/v) sugar solutions, mixed with the juice at a ratio of 1:2 and 1:3 (C1 and C2, respectively).

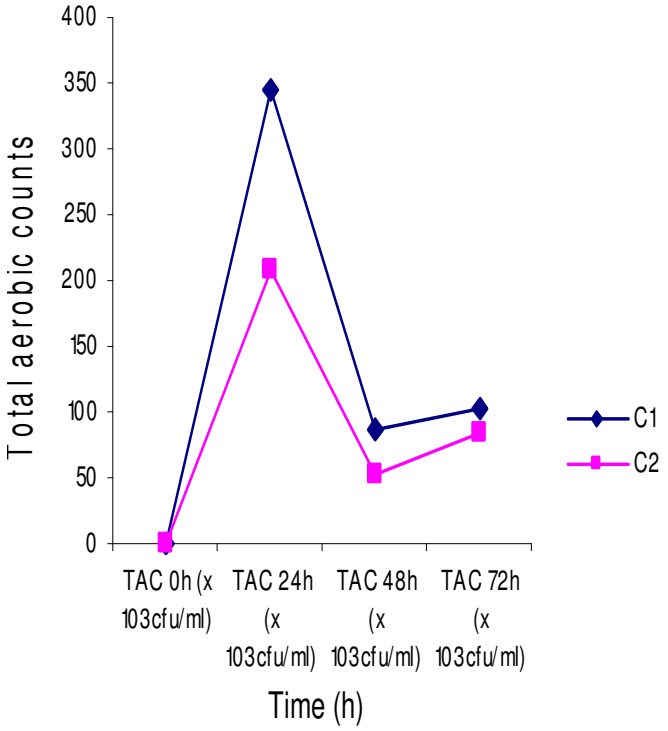

Figure 5e. Changes in % total aerobic counts with fermentation of 10% (w/v) sugar solutions, mixed with the juice at a ratio of 1:2 and 1:3 (C1 and C2, respectively).

Table 1. R_f values of wine at 48 h fermentation.

Wine	R_f value at 48 h
A1	0.51
B1	0.51
C1	0.52
A2	0.52
B2	0.51
C2	0.53

A1 = 2% (w/v) sugar activated yeast in a 1:2 yeast : fruit extract ratio;
B1 = 5% (w/v) sugar activated yeast in a 1:2 yeast : fruit extract ratio;
C1 = 10% (w/v) sugar activated yeast in a 1:2 yeast : fruit extract ratio;
A2 = 2% (w/v) sugar activated yeast in a 1:3 yeast : fruit extract ratio;
B2 = 5% (w/v) sugar activated yeast in a 1:3 yeast : fruit extract ratio;
C2 = 10% (w/v) sugar activated yeast in a 1:2 yeast : fruit extract ratio.

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Full Length Research Paper

Optimizing culture conditions for the production of endo-β-1,4-glucanase by *Aspergillus awamori* strain Vietnam Type Culture Collection (VTCC)-F099

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In the present study, twenty six strains of *Aspergillus awamori* from the Vietnam Type Culture Collection (Institute of Microbiology and Biotechnology, Vietnam University Hanoi) were used for the endoglucanase production by growing at 37°C in the growth medium. Result showed that *A. awamori* strain VTCC-F099 produced the highest level of endo β-1,4-glucanase in the growth medium, pH 6.5, at 30°C for 96 h, agitated at 200 rpm. The optimal concentration of the inducer CMC (carboxymethyl cellulose) for the endoglucanase production by *A. awamori* VTCC-F099 was 2%. Among tested carbon sources (coconut fiber, coffee shell, corncob, dried tangerine skin, peanut shell, rice bran, saw dust, sugar-cane bagasse as organic wasters and glucose, lactose sucrose as pure carbon sources), corncob showed the highest endoglucanase production by *A. awamori* VTCC-F099 at the concentration of 3%. Ammonium acetate was the best among nitrogen source (casein, peptone, fish powder, soybean powder as organic sources and CH_3COONH_4, NH_4NO_3, $(NH_4)_2SO_4$, urea as inorganic sources) for the endoglucanase production by *A. awamori* VTCC-F099 at the concentration of 0.3%.

Key words: *Aspergillus awamori,* carboxymethyl cellulose, endoglucanase production, optimization of culture conditions.

INTRODUCTION

Cellulose is a major polysaccharide constituent of plant cell walls and one of the most abundant organic compounds in the biosphere (Murai et al., 1998; Hong et al., 2001). Biological degradation of cellulose involves the synergistic action of three enzymes: Endoglucanase or carboxymethyl cellulase (CMCase) (endo β-1,4-glucanase, E.C. 3.2.1.4), exoglucanase or cellobiohydrolase (exo β-1,4-glucanase, E.C. 3.2.1.91), and β-glucosidase (β-D-glucoside glucohydrolase, E.C. 3.2.1.21) (Gielkens et al., 1999; Kang et al., 1999). Endo- β -1, 4-glucanase randomly hydrolyzes internal β-1,4-D-glycosidic bonds in cellulose producing oligos and reducing polymer length, while exo- β-1,4-glucanase cleave cellobiosyl residues from the non-reducing end of cellulose chain. Then, cellobiose is hydrolyzed by β-glucosidase to yield two glucose units (Coughlan et al., 1985).

Cellulases have a broad variety of applications in food, animal feed (Ramamurthy et al., 1987), brewing, paper pulp, and detergent (Bhat and Bhat, 1997), textile (Belghiht et al., 2001; Anish et al., 2006), fuel and chemical industries as well as waste management and pollution treatment (Mandels 1985; Ole et al., 2002). Among theses enzymes, endoglucanases have been well studied and are produced by various microbes (bacteria, yeast, and fungi), plant, and protozoans. Especially, the filamentous fungi *Aspergillus* spp. (*awamori*, *fumigatus*, *niger*, *terreus*) are preeminent in endoglucanase produc-tion (Onsori et al., 2005; Gao et al., 2008; Grigorevski-Lima, 2009). The purpose of this present work was to produce the endo-

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Abbreviations: CMC, carboxymethyl cellulose; **CMCase,** carboxymethyl cellulose.

glucanase from easily available carbon and nitrogen sources by using *A. awamori* VTCC-F099.

MATERIALS AND METHODS

Fungal strains and culture conditions

Twenty six strains of *A. awamori* from the Vietnam Type Culture Collection (Institute of Microbiology and Biotechnology, Vietnam University Hanoi) were used for the endoglucanase production by growing at 37°C in the growth medium (Mandels et al., 1976) containing (w/v): 0.03% urea, 0.14% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.03% $CaCl_2$, 0.03% $MgSO_4.7H_2O$, 0.1% peptone, 1% yeast extract, 0.1% Tween 80, 1% CMC. Trace elements were also added, using a 1% (v/v) solution of salts: 18 mM FeSO4.7H2O; 6.6 mM $MnSO_4$; 4.8 mM $ZnSO_4$, 15 mM $CoCl_2$. The initial pH of the production media was adjusted to 6.5-7.0 before sterilization.

Chemicals

Peptone and Tween 80 was purchased from Bio Basic Inc. (USA), yeast extract from Difco (USA), 3,5-dinitrosalicylic acid (DNS) from Fluka (Germany), carboxymethyl cellulose (CMC) from Prolabo (France), urea from Merck (Germany).

Endoglucanase assay

Endoglucanase activity was examined relatively by measurement of the halo diameter of enzyme diffusion on agar plates containing substrate: After 96 h of growth in the growth medium containing 0.5% (w/v) CMC, 50 µl of the culture supernatant were dropped into well on agar plates containing 0.5 % (w/v) CMC and incubated for 24 h at 4°C to diffuse enzyme. After that, the agar plates were incubated for further 24 h at 37°C (Incubator, Sanyo, Japan) and stained with 1% (w/v) lugol dye.

Endoglucanase activity was determined by Mandels et al. (1976) with 0.5% (w/v) CMC in 0.1 M potassium phosphate buffer pH 6.5. The amount of reducing sugars released in the reagent solution at 50°C for 20 min was read at the wavelength of 540 nm (spectrophotometer UV-2500, Hewlett Package, USA). Glucose was used as the standard for the estimation of reducing sugars. One unit of the endoglucanase activity was defined as the amount of enzyme required to release 1 µmol glucose per minute under experimental conditions.

Endo β-1,4-glucanase production

A. awamori VTCC-F099 was grown in 100 ml shaking flask containing 20 ml growth medium with initial pH 6.5 at 30°C, agitated at 200 rpm (Certomat HK, Sartorius, Germany). After every 24 h of cultivation, 1 ml of culture was obtained, centrifuged (MIKRO22, Hettich, Germany) and the supernatant was used to determine the endoglucanase activity as mentioned above.

Optimization of culture temperature

In order to determine the effect of culture temperature on the endo-glucanase production, *A. awamori* strain VTCC-F099 was grown in 100 ml shaking flasks containing 20 ml growth medium for 96 h at different temperatures varied from 25, 28, 30, 32, 37°C and agitated at 200 rpm.

Optimization of pH

To optimize the initial pH, *A. awamori* strain VTCC-F099 was grown in 100 ml shaking flasks containing growth medium with an initial pH range of 3 to 8.

Optimization of inducer concentration

To investigate the effect of CMC as an inducer on the endoglucanase production, *A. awamori* strain VTCC-F099 was grown in 100 ml shaking flasks containing 20 ml growth medium, pH 6.5 added with CMC at various concentrations ranging from 0.2 to 4% (w/v).

Optimization of carbon source and its concentration

To optimize the carbon source, *A. awamori* strain VTCC-F099 was grown in 100 ml shaking flasks containing 20 ml growth medium except yeast extract, which was substituted by another carbon source (coconut fiber, coffee shell, corncob, dried tangerine skin, peanut shell, rice bran, saw dust, sugar-cane bagasse, glucose, lactose, sucrose, yeast extract). After determination of the carbon source for the maximal endoglucanase production, *A. awamori* strain VTCC-F099 was grown in the growth medium containing corncob as the optimum carbon source at different concentrations ranging from 0.5 to 5% (w/v).

Optimization of nitrogen source and its concentration

To optimize the nitrogen source, *A. awamori* strain VTCC-F099 was grown in 100 ml shaking flasks containing 20 ml with carbon source optimized growth medium and one of various nitrogen sources including ammonium acetate, ammonium sulfate, casein, fish powder, peptone, soybean powder, and urea. After determination of the optimal nitrogen source, *A. awamori* strain VTCC-F099 was grown in 100 ml shaking flasks containing 20 ml optimal growth medium with an initial pH 6.5, with 3% corncob, and ammonium acetate at concentrations ranging from 0.1 to 0.55% (w/v).

RESULTS AND DISCUSSION

Screening *A. awamori* strains producing endoglucanase

The endoglucanase production by 26 filamentous *A. awamori* strains from Vietnam Type Culture Collection was examined; all of the strains showed endoglucanase activity on the agar plates containing CMC as the substrate. Among them, four strains showed the largest helo of clearing zone on CMC containing plates including *A. awamori* VTCC-F099 (29 mm), VTCC-F245 (28 mm), VTCC-F261 (27 mm), VTCC-F350 (26 mm) (Table 1). Among these fours strains, *A. awamori* VTCC-F099, VTCC-F245, and VTCC-F350 showed the highest levels of endoglucanase production in submerged fermentation, with an endoglucanase activity of 0.513, 0.489, and 0.484 U/ml, respectively. *A. awamori* strain VTCC-F099 was selected for optimization of the endoglucanse production (Figure 1).

Table 1. Endoglucanase production by 26 *A. awamori* strains. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

A. awamori strain	Diameter of clearing zone (mm)	CMCase activity (U/ml)	*A. awamori* strain	Diameter of clearing zone (mm)	CMCase activity (U/ml)
VTCC-F-014	12.5 ± 0.35	0.42 ± 0.020	VTCC-F-261	27 ± 0.30	0.33 ± 0.027
VTCC-F-020	12.5 ± 0.42	0.38 ± 0.011	VTCC-F-262	19 ± 0.34	0.33 ± 0.017
VTCC-F-061	15 ± 0.21	0.45 ± 0.035	VTCC-F-269	15.5 ± 0.45	0.23 ± 0.010
VTCC-F-062	13.5 ± 0.23	0.46 ± 0.065	VTCC-F-270	9.5 ± 0.48	0.38 ± 0.020
VTCC-F-063	15 ± 0.32	0.38 ± 0.025	VTCC-F-296	4 ± 0.50	0.34 ± 0.018
VTCC-F-064	18 ± 0.41	0.42 ± 0.042	VTCC-F-311	13 ± 0.56	0.40 ± 0.021
VTCC-F099	29 ± 0.35	0.51 ± 0.059	VTCC-F-312	14 ± 0.48	0.39 ± 0.033
VTCC-F-100	17.5 ± 0.12	0.45 ± 0.040	VTCC-F-317	15.5 ± 0.10	0.41 ± 0.018
VTCC-F-135	15.5 ± 0.05	0.43 ± 0.124	VTCC-F-350	26 ± 0.46	0.48 ± 0.007
VTCC-F-207	15 ± 0.25	0.33 ± .0120	VTCC-F-353	19 ± 0.70	0.36 ± 0.023
VTCC-F-229	18.5 ± 0.16	0.47 ± 0.021	VTCC-F-356	15 ± 0.59	0.39 ± 0.040
VTCC-F-245	28 ± 0.57	0.49 ± 0.069	VTCC-F-401	16 ±0.32	0.46 ± 0.057
VTCC-F-259	14 ± 0.43	0.44 ± 0.020	VTCC-F-406	14.5 ± 0.21	0.46 ± 0.028

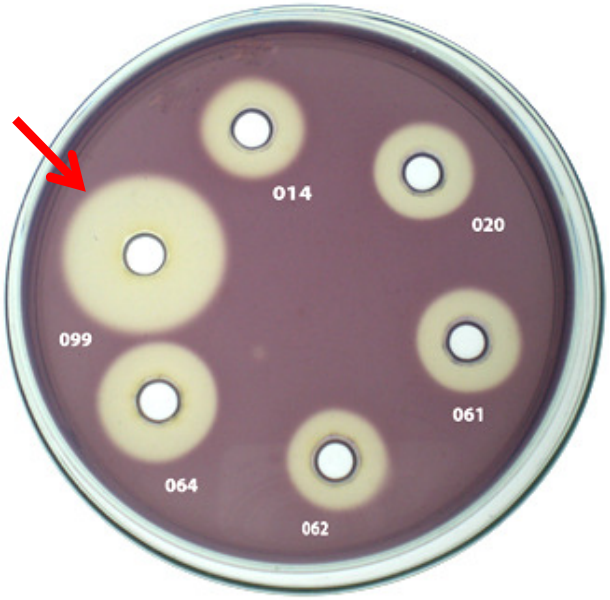

Figure 1. Endoglucanase activity on agar plates containing 0.5% (w/v) CMC and staining with lugol. 014, 020, 061, 062, 064, 099, are abbreviated for *A. awamori* VTCC-F014, *A. awamori* VTCC-F020, *A. awamori* VTCC-F061, *A. awamori* VTCC-F062, *A. awamori* VTCC-F064, and *A. awamori* VTCC-F099.

Endo β-1,4-glucanase production course

The endoglucanase production by *A. awamori* VTCC- F099 increased gradually from 0.41 U/ml (74%) at 24 h of cultivation to the maximum of 0.55 U/ml (100%) at 96 h of cultivation (Figure 2). Then the endoglucanase production decreased strongly to 0.39 U/ml (71%) at 120 h of cultivation and remained constant 0.39 - 0.33 U/ml (71 to 60%) in a long time interval from 120 to 240 h of cultivation (Figure 2).

The highest levels of endoglucanase production by *A. fumigatus* corresponded to 0.365 U/ml and was obtained using sugarcane bagasse (1%) and corn steep liquor (1.2%) in submerged fermentation within 6 days of cultivation (Grigorevski-Lima, 2009).

Optimization of cultivation temperature

The endoglucanase production by *A. awamori* VTCC-F099 was maximum (0.551 U/ml) at 30°C (Figure 3). The optimum temperature for endoglucanase production by *A. fumigatus* (Grigorevski-Lima, 2009) was also 30°C.

Optimization of initial medium pH

A. awamori VTCC-F099 produced the highest levels of endoglucanase (5.22 U/ml) at the initial medium pH 6.5 (Figure 4). At the lower (pH 3) or higher (pH 8) initial medium pH, the endoglucanase production was also very high 4.59 U/ml (88%) and 3.65 U/ml (70%) in comparison to the maximum at the initial pH of 6.5, respectively.

Optimization of inducer concentration

The CMC is a substrate for endoglucanases and showed an induction effect on the endoglucanase production. The addition of CMC at the concentration from 0.2 to 2% to the growth medium increased the endoglucanase production gradually from 0.17 U/ml (21%) to the maximum 0.81 U/ml. The addition of more CMC (at the concentration of 2.5 - 4%) to the culture medium decreased the endoglucanase production gradually to

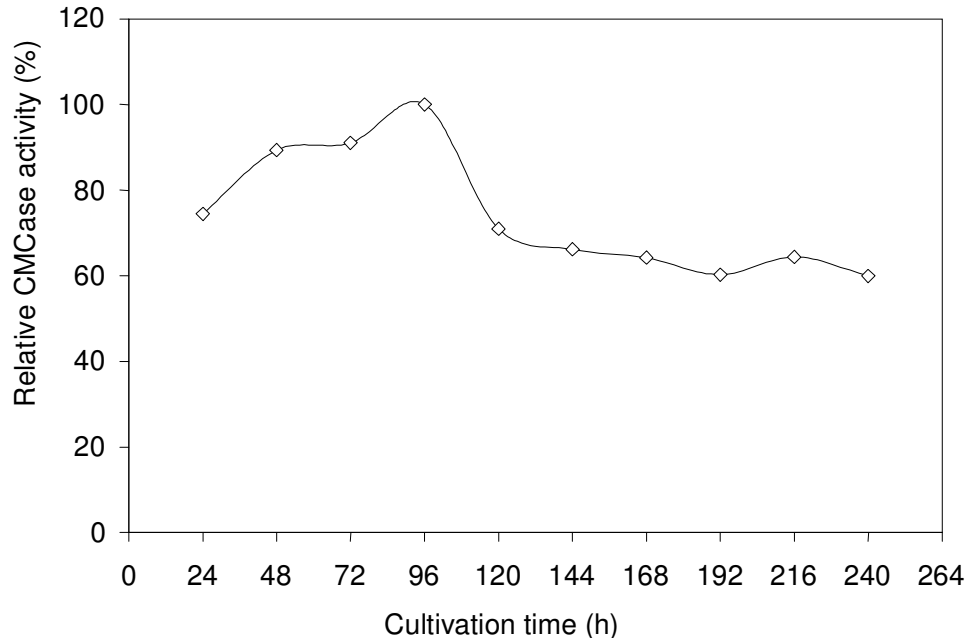

Figure 2. Time course of the endoglucanase production by *A. awamori* VTCC-F099. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

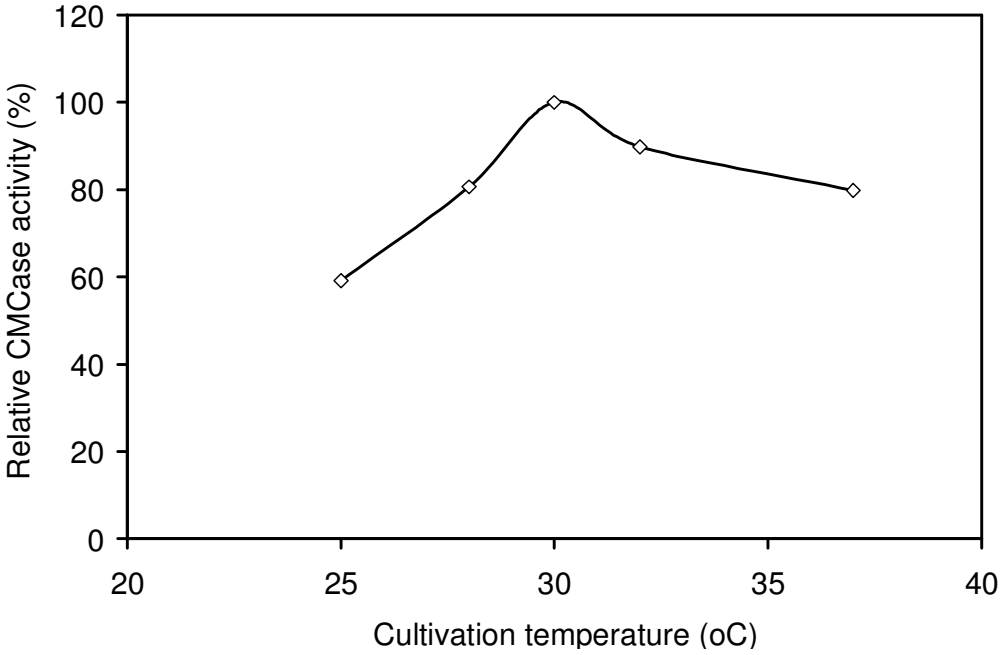

Figure 3. Effect of cultivation temperature on endoglucanase production by *A. awamori* VTCC-F099. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

0.42 U/ml (52%). No addition of CMC showed a similar effect on endoglucanase production as the addition of 0.2% CMC, just 0.15 U/ml (18%) in comparison to the maximum production (Figure 5).

Optimization of carbon source and its concentration

Among the investigated carbon sources, corncob was the most appropriate carbon source for the endoglucanase

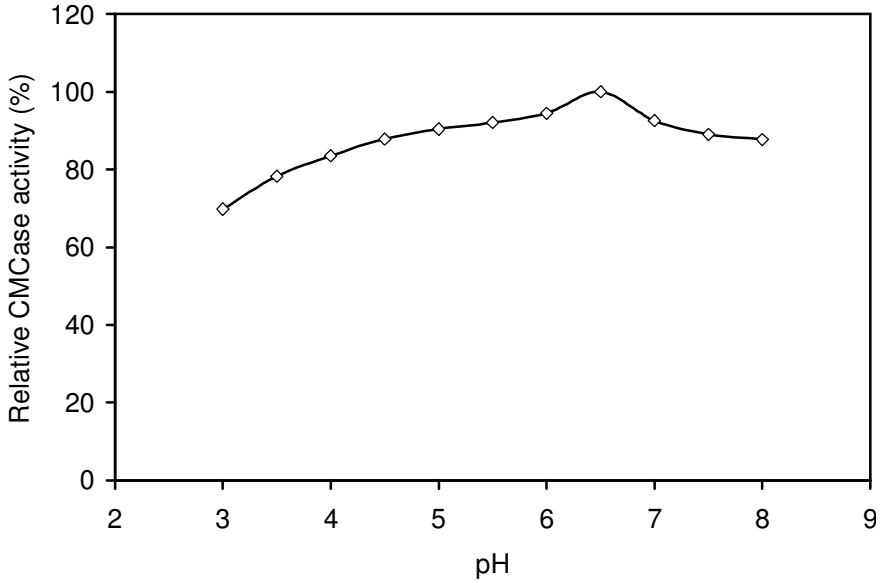

Figure 4. Effect of initial pH of culture medium on endoglucanase production by *A. awamori* VTCC-F099. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

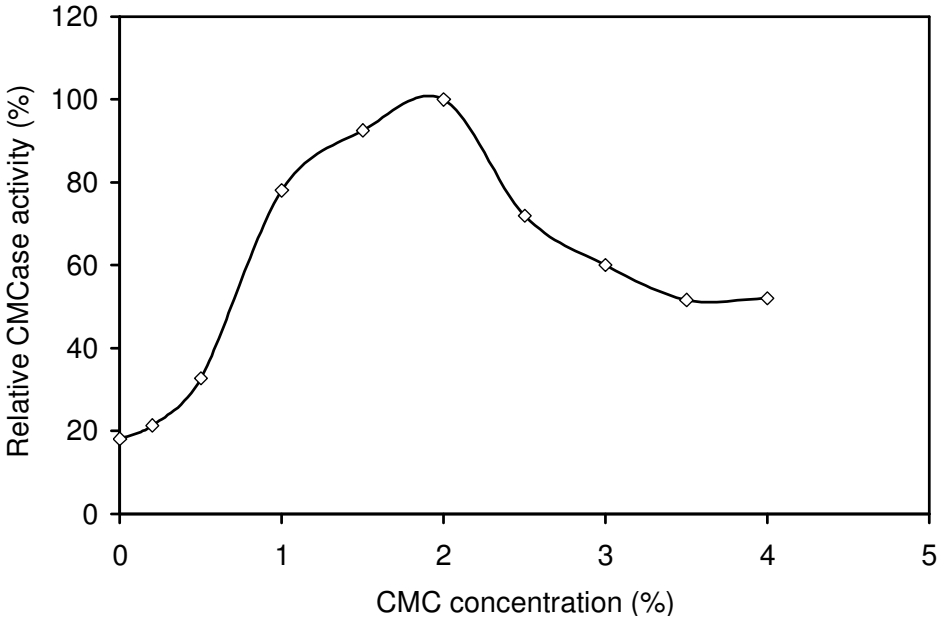

Figure 5. Effect of CMC concentration on endoglucanase production by *A. awamori* VTCC-F099. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

production (0.868 U/ml, 100%) by *A. niger* VTCC-F099 (Table 2). Other carbon sources produced the enzyme at the level of 34-85% in comparison to corncob as carbon source (Table 2). Sugar-cane bagasse was a suitable carbon source for the endoglucanase production by *A. fumigatus* strain (Grigorevski-Lima, 2009).

The endoglucanase production by *A. awamori* VTCC-F099 increased from 0.64 U/ml (71%) in the growth medium containing 0.5% corncob to the maximum 0.90 U/ml in the growth medium containing 3% corncob (Figure 6). The endoglucanase production decreased gradually to 0.53 U/ml (59%) in the growth medium containing 5% corncob. The highest level of endoglucanase by *A. fumigatus* was obtained using 1% sugarcane

Table 2. Effect of carbon source on endoglucanase production by *A. awamori* VTCC-F099. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

Carbon source	Endoglucanase activity	
	U/ml	%
Coconut fiber	0.521 ± 0.046	53
Coffee shell	0.643 ± 0.125	74
Corncob	0.868 ± 0.233	100
Dried mandarin skin	0.511 ± 0.055	59
Glucose	0.686 ± 0.146	79
Lactose	0.295 ± 0.087	34
Peanut shell	0.553 ± 0.117	64
Rice bran	0.554 ± 0.048	64
Sucrose	0.607 ± 0.038	70
Saw dust	0.411 ± 0.089	42
Sugarcane bagasse	0.734 ± 0.196	85
Yeast extract	0.652 ± 0.212	75

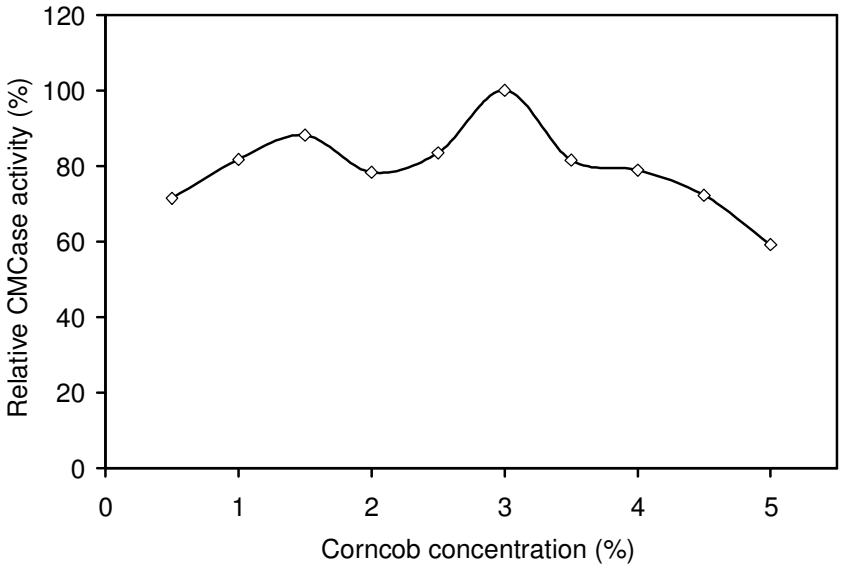

Figure 6. Effect of corncob concentration on endoglucanase production by *A. awamori* VTCC-F099. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

ugarcane bagasse (Grigorevski-Lima, 2009).

Optimization of nitrogen source and its concentration

Among the examined nitrogen sources (Table 3), ammonium acetate was the best nitrogen source for the endoglucanase production by *A. awamori* VTCC-F099 (4.85 U/ml). The enzyme production in growth medium containing other nitrogen sources was obtained only (70 - 81%) in comparison to ammonium acetate (Table 3). *A. awamori* VTCC-F099 produced the highest levels of endoglucanase amount (4.98 U/ml) when ammonium acetate was used with the amount of 0.3% (Figure 7). The lowest (0.1%) and highest (0.55%) amount of ammonium acetate in the growth medium reduced the endoglucanase production to 71% (3.53 U/ml) and 68% (3.39 U/ml) in comparison to the optimal amount of ammonium acetate 0.3%.

Conclusion

In conclusion, *A. awamori* VTCC-F099 is capable of

Table 3. Effect of nitrogen source on endoglucanase production by *A. awamori* VTCC-F099. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

Nitrogen source	Endoglucanase activity	
	U/ml	%
Ammonium acetate	4.847 ± 0.189	100
Ammonium sulfate	4.187 ± 0.124	87
Casein	3.440 ± 0.128	71
Fish powder	4.101 ± 0.118	85
Peptone	4.101 ± 0.150	75
Soybean powder	3.508 ± 0.115	72
Urea	3.866 ± 0.077	80

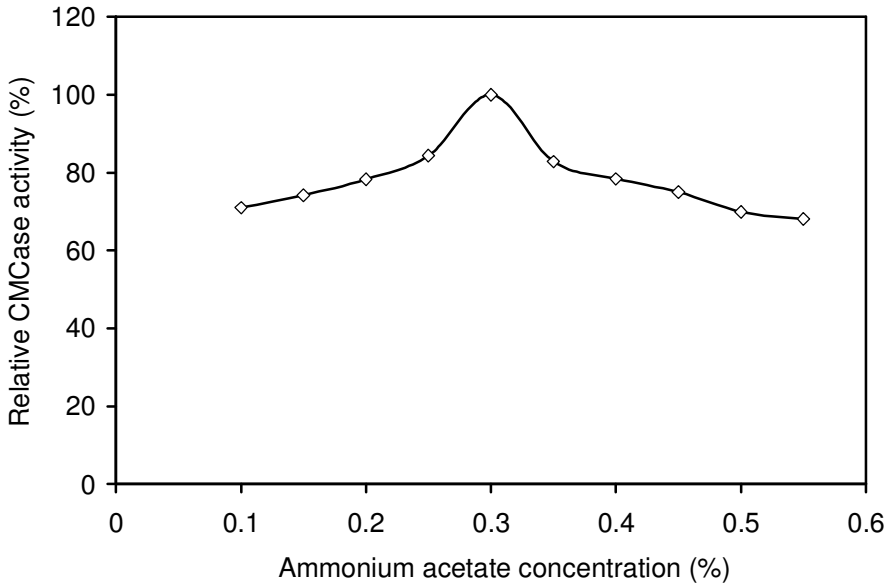

Figure 7. Effect of ammonium acetate concentration on endoglucanase production by *A. awamori* VTCC-F099. The endoglucanase activity was determined with 0.5% CMC in 0.1 M potassium phosphate buffer pH 6.5 at 30°C.

producing endoglucanase from corncob and ammonium acetate. The endoglucanase production from corncob and ammonium acetate was highest at 96 h of production, with an initial pH 6.5 and at 30°C.

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Full Length Research Paper

Screening for the optimal induction parameters for periplasmic producing interferon-α2b in *Escherichia coli*

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Screening for optimum induction parameters to improve the production of periplasmic interferon-α2b (PrIFN-α2b) by recombinant *Escherichia coli* was conducted using shake flask culture. Recombinant *E. coli* Rosetta-gami 2(DE3) harboring the plasmid pET26b containing IFN-α2b gene under the control of the T7*lac* promoter was used, where the induction was accomplished by isopropyl β-D-1-thiogalactopyranoside (IPTG). The induction parameters (inducer concentration, point of induction, induction temperature and the length of induction) were analyzed to find the suitable range to be used for further optimization process. From the analysis, narrow range of induction temperature from 16 to 30°C and IPTG lower than 2 mM were found suitable for induction of PrIFN-α2b. On the other hand, early log phase was the preferred time to initiate the induction and the length of induction was dependent on the combination of other induction parameters used.

Key words: Interferon-α2b (IFN-α2b), induction parameter, *Escherichia coli,* periplasm, shake flask culture.

INTRODUCTION

Human interferon-α2b (IFN-α2b) is a member of the cytokine family with the ability to induce antiproliferative, antiviral and immunomodulating activities. It has been approved for the treatments of gastrointestinal tract diseases, various cancers including hairy cell leukemia, Acquired Immunodeficiency Syndrome (AIDS)-related Kaposi's sarcoma, chronic myelogenous leukemia, chronic hepatitis B and cirrhosis (Pestka et al., 2004; Billiau, 2006; Chelbi-Alix and Wietzerbin, 2007). For a massive therapeutic use of IFN- α2b, large quantities of this cytokine are currently produced in recombinant strains of *Escherichia coli* and other genetically modified hosts (Pimienta et al., 2002; Srivastava et al., 2005; Ghosalkar et al., 2008).

The *E. coli* recombinant protein expression system has been, and is still the system of choice for the production of IFN-α2b. Indeed, IFN-α gene do not have introns (Baron and Narula, 1990). Targeting the recombinant protein into periplasmic space of *E. coli* offer several advantages. For example, N-terminal amino acid residue of the secreted product can be identical to that of the naturally secreted gene product, and protease activity is considered to be much lower in the periplasmic space than in the cytoplasm. Thus, production in periplasmic space may avoid problem related to protein degradation. Since the periplasm contains far fewer native host proteins, purification of target protein could be simplified (Schumann and Ferreira, 2004; Choi et al., 2006; Terpe, 2006).

Generally, foreign protein expression causes a metabolic burden on the cell which may reduce the growth

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Abbreviations: PrIFN-α2b, Periplasmic interferon-α2b; **IPTG,** isopropyl β-D-1-thiogalactopyranoside.

rate, cell yield, product expression and plasmid stability. In addition, environmental factor such as temperature also significantly affect the cell metabolism (Bronikowski et al., 2001; Han and Lee, 2006). Furthermore, environmental factors also have a pronounced effect on protein folding and stability during protein expression, and frequently studied to improve solubility of the proteins (Donovan et al., 1996; Weickert et al., 1996). The level of isopropyl β-D-1-thiogalactopyranoside (IPTG) used to induce protein expression can be varied to adjust the extent of metabolic burden imposed on the cell (Donovan et al., 1996; Hansen et al., 1998). Since IPTG is not metabolized, it permits the separation of the physiological function of lactose as a carbon source from its function in the regulation of lac gene expression. These properties make IPTG ideal in industrial applications for the large scale production of expressed proteins. However, strong induction may cause the formation of inclusion bodies, which are inactive (Kopetzki et al., 1989).

Strong induction may also increase the death rate of host cells (Donovan et al., 1996). The maximum yield of foreign protein from the fermentation process is depended on the point in the growth cycle at which expression is induced and also the length of induction (Donovan et al., 1996). Some recombinant strains are highly influenced by the induction; where growth and viability is drastically reduced following the induction, especially periplasmic expression (Balagurunathan and Jayaraman, 2008). Longer induction time may also cause exhaustion to nutrients available in the culture (Chen and Morgan, 2006). For this reason, it is important to balance the induction capacity as well as protein production based on the environmental condition employed during the expression of target protein.

Therefore, the objective of this study is to analyze the effect of different environmental factors during induction such as temperature, IPTG concentration, point of induction and length of induction on growth of recombinant *E. coli* and the expression of periplasmic interferon-α2b (PrIFN-α2b). The information gathered may be used for optimization of the fermentation process, which aims at improving the production of recombinant protein in the periplasmic space.

MATERIALS AND METHODS

Microorganism

E. coli strain Rosetta-gami 2(DE3) (Cat. No. 71351, Novagen, USA) harboring pET-26b-IFN was used for the production of recombinant PrIFN-α2b. The details of the preparation of this strain are described in Ramanan et al. (2010a). The stock cultures were kept at -20 and -80°C in 10% (v/v) glycerol, for immediate and long-term use.

Medium and fermentation

The culture medium consisted of 47.6 g/l terrific broth (TB) (Cat. No. 71754-3, Merck, USA), 4 g/l glycerol (Cat. No. 356352, Merck, USA), 30 mg/l kanamycin (Cat. No. K4378, Sigma-Aldrich, USA) and 34 mg/l chloramphenicol (Cat. No. 220551, Merck, USA) which were used in this study.

The inoculum was prepared by inoculating 1% (v/v) of stock culture into 50 ml Falcon tube containing 10 ml medium. The tube was incubated at 37°C in incubator shaker, agitated at 250 rpm for 24 h and this culture was used as standard inoculum for all fermentations. The flasks were seeded with 2% (v/v) inoculum and then incubated at 37°C on a rotary shaker (Certomat, B. Braun, Germany) agitated at 250 rpm. All the fermentations were conducted in 250 ml Erlenmeyer flask containing 50 ml medium. During the fermentation, different concentrations of IPTG were added to the culture at different fermentation times according to the need of each experiment.

Experimental design

The screening for suitable induction strategy was carried out by analyzing the suitable range of individual effect on the production of recombinant PrIFN-α2b in periplasmic *E. coli*. The strength of induction was examined using different IPTG concentrations, followed by investigating the suitable induction point and induction temperature. The combinations of lowest and highest factor of each variable were used to analyze the suitable length of induction time for the maximum production of PrIFN-α2b.

In order to investigate the effect of IPTG concentration, the cultures were induced after 2 h of fermentation with different concentrations of IPTG (ranging from 0.1 to 3 mM) for 20 h of induction time. The effect of induction point was studied by inducing the fermentation cultures at two points, which represents early and late-log phase that corresponds to cell density of ~0.6 and 9 measured at A_{600}, respectively. IPTG with a concentration 0.1 mM was added to the cultures and the fermentation was continued until 12 h of induction time at 37°C.

To investigate the effect of different induction temperatures on periplasmic production, the recombinant *E. coli* was first cultured at 37°C. After 2 h, the cultures were induced with 0.1 mM IPTG and then incubated at different induction temperatures (16, 30 and 37°C) up to 22 h of cultivation. To screen the suitable length of induction, 8 sets of interactions of all induction strategies (IPTG, point of induction and induction temperature) were performed in several shake flask cultures. All fermentations were carried out in triplicates. During the fermentation, samples were withdrawn at time intervals and stored at -20°C prior to analysis.

Analytical procedure

The harvested culture samples were centrifuged at 10,000 ×g (Micro 22R, Hettich Zentrifugen, Germany) for 20 min to obtain the cell pellets for protein extraction. The protein fraction from periplasmic space was extracted using osmotic shock method as described by Ramanan et al. (2009). Briefly, the cell pellets were resuspended in ice cold water and incubated for 5 - 15 min and then centrifuged for 20 min at 10,000 ×g, at 4°C. The cell pellets were then resuspended in 1 ml osmotic shock solution, containing 20% (w/v) sucrose, 33 mM Tris-HCl (pH 8.0) and 0.5 mM EDTA. The cell suspension was vortexed and then centrifuged at 10,000 ×g, at 4°C. The harvested shrunk cells were re-suspended in ice-cold water and incubated at room temperature for 5 - 15 min with shaking. The periplasmic protein was recovered after separating the cells from the solution by centrifuging at 10,000 ×g for 20 min.

The samples were analyzed for total protein content using Bradford method (Bradford, 1976) according to Bio-Rad protein assay kit manual (Bio-Rad, USA). Bovine serum albumin (BSA) was used as a standard in a linear range (0.1 to 0.5 mg/ml). The amount

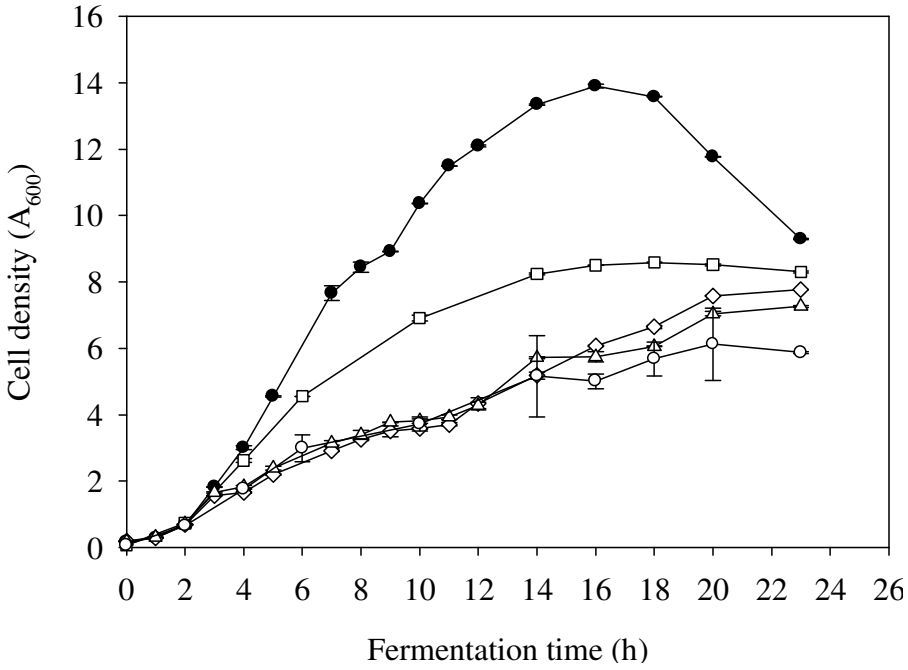

Figure 1. Growth profiles of recombinant *E. coli* when induced with different IPTG concentration at A_{600} 0.6 at 37°C. [●] not induced; [□] 0.1 mM IPTG; [◊] 1 mM IPTG; [Δ] 2 mM IPTG; and [○] 3 mM IPTG. The error bar represents the standard deviation.

of PrIFN-α2b was measured using biosensor chip in Biacore3000 as described in our previous study. The biosensor chip was prepared as described elsewhere (Ramanan et al., 2010b, Ramanan et al., 2009).

RESULTS

Effect of IPTG concentration

The effect of different IPTG concentrations on growth of recombinant *E. coli* is shown in Figure 1. After the induction, growth rate of recombinant *E. coli* was reduced and severe reduction was observed when higher IPTG concentration was used. The effect of different IPTG concentrations on the production of periplasmic protein, PrIFN-α2b and specific product yield are shown in Table 1. At 8 h of induction time, 0.1 mM IPTG yielded the highest PrIFN-α2b production as well as the secretion of periplasmic protein. When the concentration of IPTG was increased from 0.1 to 3 mM, production of both periplasmic protein and PrIFN-α2b was greatly reduced. On the other hand, the highest specific product yield was obtained at 4 h of induction using 1 mM IPTG. The reduction of periplasmic protein, PrIFN-α2b and specific product yield were observed after 4 h of induction time at 1 and 3 mM of IPTG. On the other hand, similar reductions of all the responses were observed after 8 h of induction time at 0.1 mM of IPTG. From the results of this study, it can be concluded that the production of PrIFN-α2b was inhibited at higher IPTG concentrations.

Effect of induction point

Induction point is the point at which the target protein starts to express. Several aspects such as the ability of host cells to produce protein and the availability of protein expression tools need to be considered in the initiation of protein expression. Effect of induction point, early- and late- log phase, on growth of recombinant *E. coli* and the production of PrIFN-α2b is shown Figure 2. At early-log phase induction, the profile of growth rate, periplasmic protein, PrIFN-α2b and specific product yield were in the increasing trend until the end of fermentation. Furthermore, the level of periplasmic protein, PrIFN-α2b and specific product yield were higher at early-log phase induction than at late-log phase induction.

At late-log phase induction, periplasmic protein PrIFN-α2b and specific product yield were drastically dropped towards the end of fermentation. However, the final growth obtained during late-log phase induction was higher than at the early-log phase induction.

Effect of induction temperature

The influence of induction temperature (from 16 to 37°C) on growth of recombinant *E. coli* and PrIFN-α2b production is shown in Figure 3. Growth of recombinant *E. coli* was reduced when the cultivation temperature was reduced from 37 to 16°C (Figure 3A). Growth was greatly inhibited at low temperature (16°C). The highest PrIFN-

Table 1. Production of PrIFN-α2b and periplasmic protein when induced with different IPTG concentrations at A_{600} ~0.6 at 37°C.

Parameter	IPTG concentration (mM)	Post induction time (h)		
		4	8	12
PrIFN-α2b (ng/ml)	0.1	18.28 (0.40)	49.04 (1.30)	23.75 (1.17)
	1	22.58 (1.31)	20.32 (0.49)	4.87 (1.32)
	2	21.66 (1.92)	10.36 (0.76)	6.54 (0.81)
	3	3.24 (0.37)	2.66 (0.29)	3.43 (0.16)
Periplasmic Protein (mg/ml)	0.1	0.07 (0.01)	0.30 (0.05)	0.168 (0.03)
	1	0.06 (0.02)	0.09 (0.02)	0.120 (0.03)
	2	0.07 (0.03)	0.09 (0.01)	0.101 (0.01)
	3	0.03 (0.01)	0.05 (0.01)	0.209 (0.01)
Specific product yield (µg/g)	0.1	7.46	12.62	5.05
	1	32.93	8.68	1.20
	2	19.21	5.19	2.07
	3	2.16	1.37	1.19

The samples were taken at different time intervals (4, 8 and 12 h after induction) from each culture induced with different IPTG concentrations. The value in bracket is the standard deviation. Specific product yield were calculated with the average values.

α2b was produced at 30°C, while production of PrIFN-α2b was very low at 16 and 37°C (Figure 3B). Although the production of PrIFN-α2b and protein was maintained at high level for cultivation at 30°C, towards the end of fermentation (at 12 h after induction), the yield at 16°C was increased rapidly and yielded higher specific productivity than at 30°C. This is because at lower temperature, the expressed protein tends to be in soluble form and also tend to transfer across the membrane at a higher level. To improve the specific product yield for fermentation with induction temperature at 16°C, the induction time was extended.

Length of induction time

Eight sets of fermentation condition involving the interaction of each of the induction strategy was analyzed to obtain the suitable length of induction time for maximum production of PrIFN-α2b. The growth profiles of each culture during the fermentation along with the total periplasmic protein and PrIFN-α2b production for early- and late-log phase induction are shown in Figures 4 and 5, respectively. Different combinations of induction condition gave different results. For induction at early-log phase, production of PrIFN-α2b was increased until the end of cultivation irrespective of the difference in temperature and induction strength. However, for induction at late-log phase, the production of PrIFN-α2b was decreased at certain point after induction and it depended on the temperature. For instance, the drop in the production of PrIFN-α2b was noticed after 8 h of induction at low temperature (16°C) (Figures 5a and b) and after 4 h at high temperature (37°C) (Figures 5b and d). The induction strength was attributed to the level of expression in all conditions. As described above, the total periplasmic protein profile followed similar trend of growth profile in all experiments indicating that the secretion is associated with cell growth.

DISCUSSION

In order to screen the suitable induction parameters for enhancement of recombinant protein production, several aspects need to be considered. One of the most important factors is induction strength. The induction strength was not necessarily high as it could cause metabolic burden to the growing cells, and hence would reduced the yield. The normal range of induction strength used for protein expression ranged from 0.005 to 5 mM IPTG. However, 1 mM IPTG was widely used (Donovan et al., 1996). For example, to induce IFN-a2b production as inclusion bodies in *E. coli* 1 mM IPTG has been used (Srivastava et al., 2005; Valente et al., 2006). On the other hand, 2 mM of IPTG was used to induce IFN-α2b production in peri-plasmic *E. coli* (Barbero et al., 1986). In our case, the use of IPTG at concentration higher than 1 mM greatly influenced the specific product yield and the total amount of product. Although 1 mM IPTG gave the highest speci-fic product yield, the cell growth was inhibited which in turn, reduced the final amount of the targeted protein.

Another induction factor that influenced the production of recombinant protein in periplasmic *E. coli* was the point at which the induction was made. Commonly, the optimum point for induction was at mid-log phase (Peng et al., 2004; Vásquez-Bahena et al., 2006). In some instance,

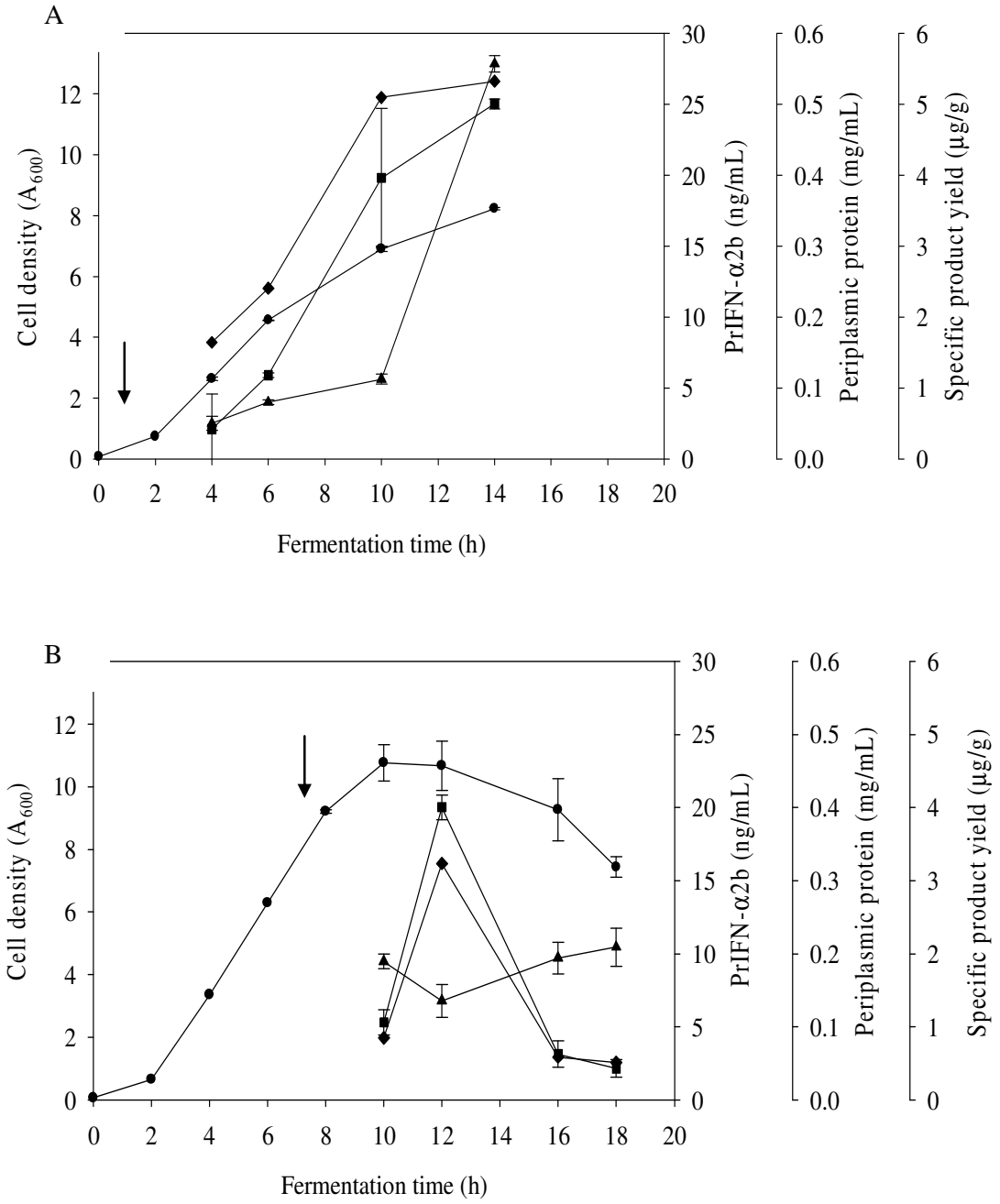

Figure 2. Production of PrIFN-α2b and periplasmic protein when induced at different induction point. A. Early log phase (A_{600} ~0.6); B. late log phase (A_{600} ~9) with 0.1 mM IPTG at 37°C; [●] cell density; [■] PrIFN-α2b; [▲] periplasmic protein; [♦] specific product yield. The error bar represents the standard deviation and the arrow represents induction starting point.

induction was also performed at a stationary phase (Yildir et al., 1998). Indeed, the induction point would depend on the response of strains during the induction. Strains whose growth and/or viability are drastically reduced after induction, late-log or stationary phase induction would provide high cell densities, which in turn, increased the production of the required protein. In this case, the addition of IPTG at early-log phase might utilized the cellular machinery for the foreign protein expression which reduced the production of protein required for cell proliferation (Donovan et al., 1996).

Sandén et al. (2003) analyzed the limiting factors in *E. coli* when induced at different induction points with respect to specific growth rate. They found that, the ribosome (represented by rRNA) was degraded upon induction at high specific growth rate due to high production level. At

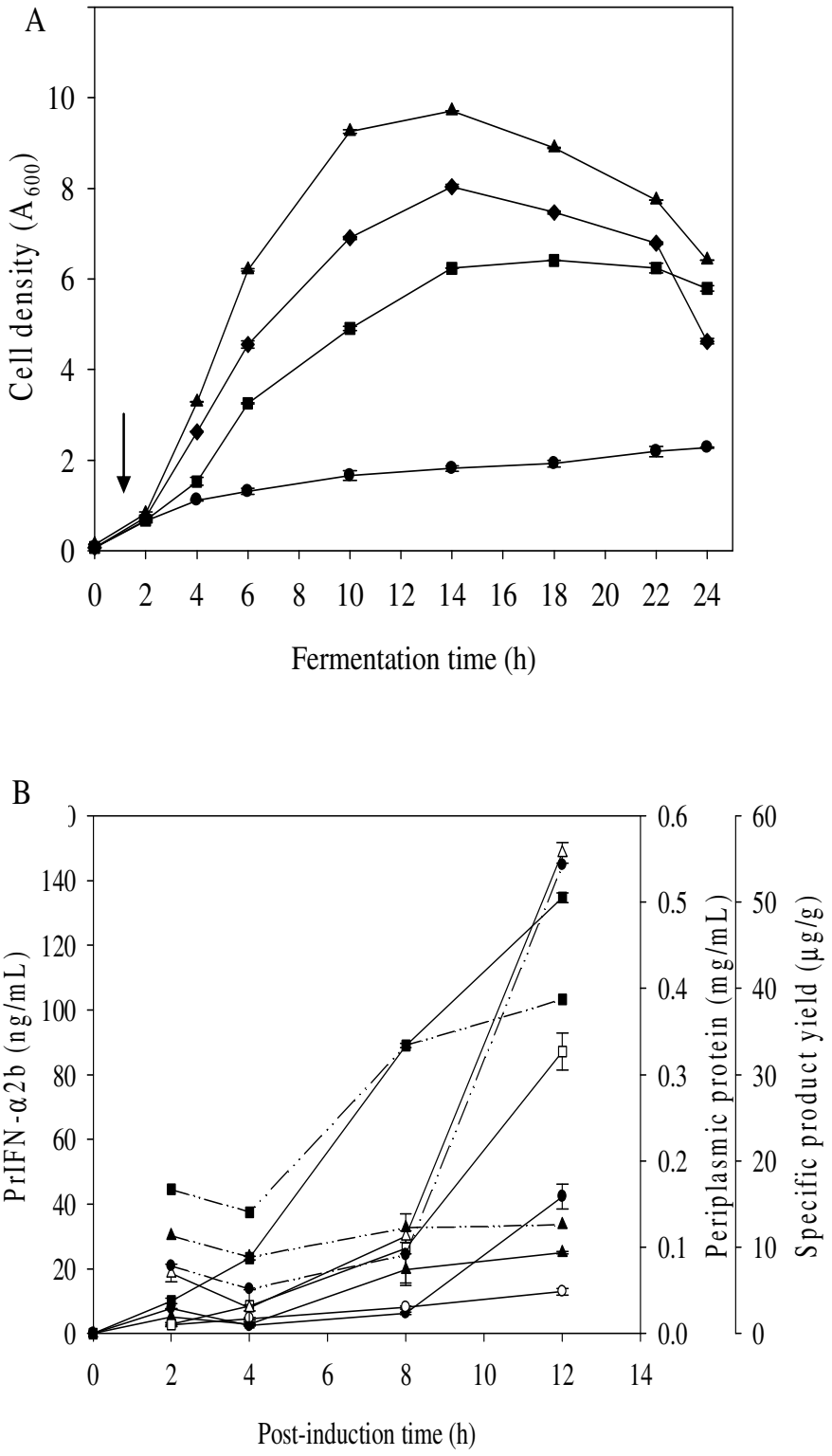

Figure 3. Effect of temperature on A. Growth of recombinant *E. coli* RG 2 (DE3); B. PrIFN-α2b production for culture, initially cultivated at 37°C, induced with 0.1 mM IPTG after 2 h of cultivation. Symbols for A: [●] induced at 16°C; [■] induced at 30°C; [♦] induced at 37°C; [▲] normal growth without induction; B.: [●] PrIFN-α2b at 16°C; [■] PrIFN-α2b at 30°C; [▲] PrIFN-α2b at 37°C; [○] protein at 16°C; [□] protein at 30°C; [Δ] protein at 37°C. The line of dash-dot-dot represent specific product yield, with symbols: [●] PrIFN-α2b at 16°C; [■] PrIFN-α2b at 30°C; [▲] PrIFN-α2b at 37°C. The error bar represents the standard deviation and the arrow indicates the induction starting point.

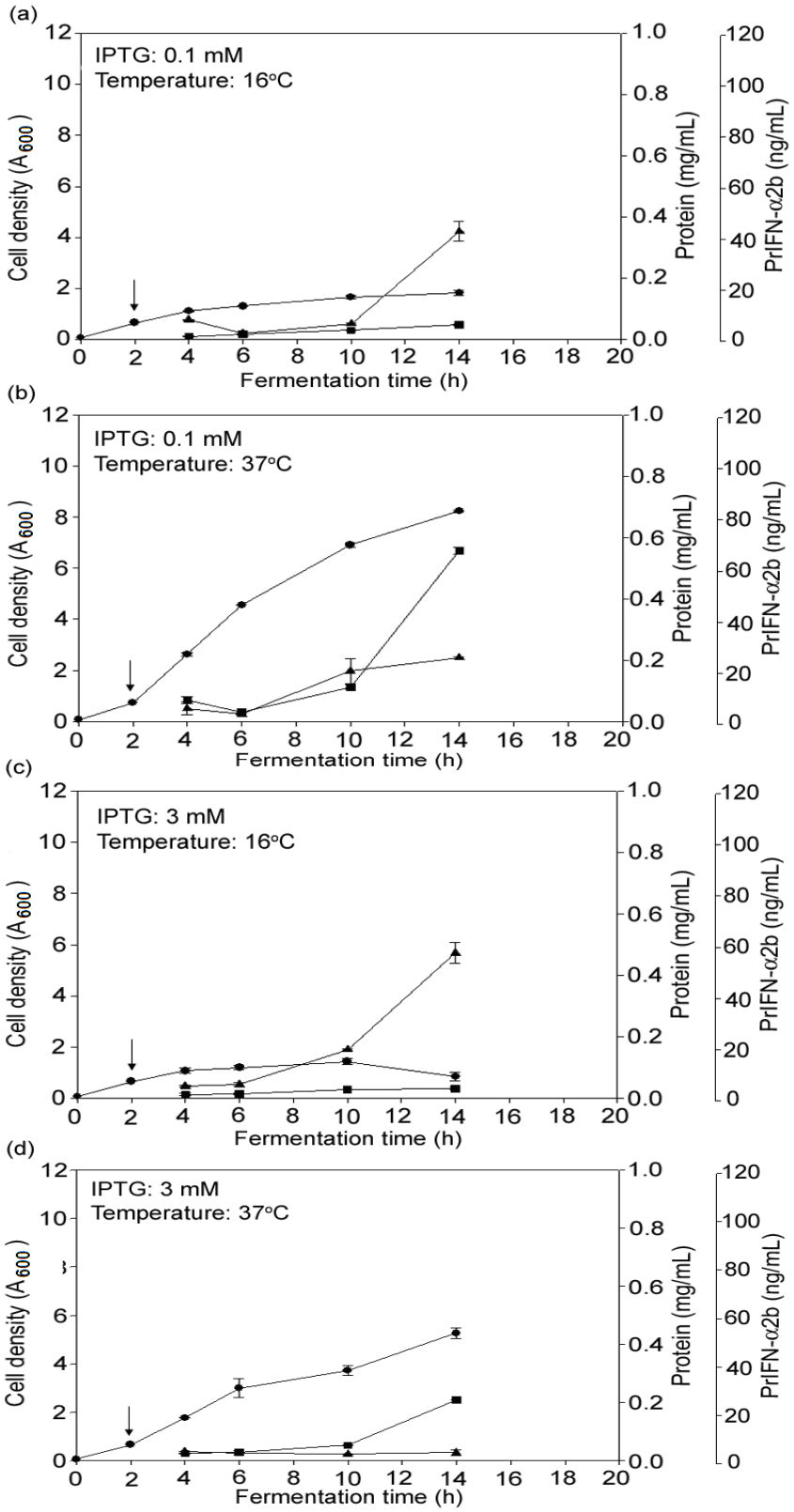

Figure 4. Growth profile of *E. coli* and production of PrIFN-α2b and periplasmic protein during fermentation when induced at early log phase with different IPTG concentrations and induction temperatures. [●] Cell density; [■] protein; [▲] PrIFN-α2b. The error bar represents the standard deviation and the arrow represents induction starting point.

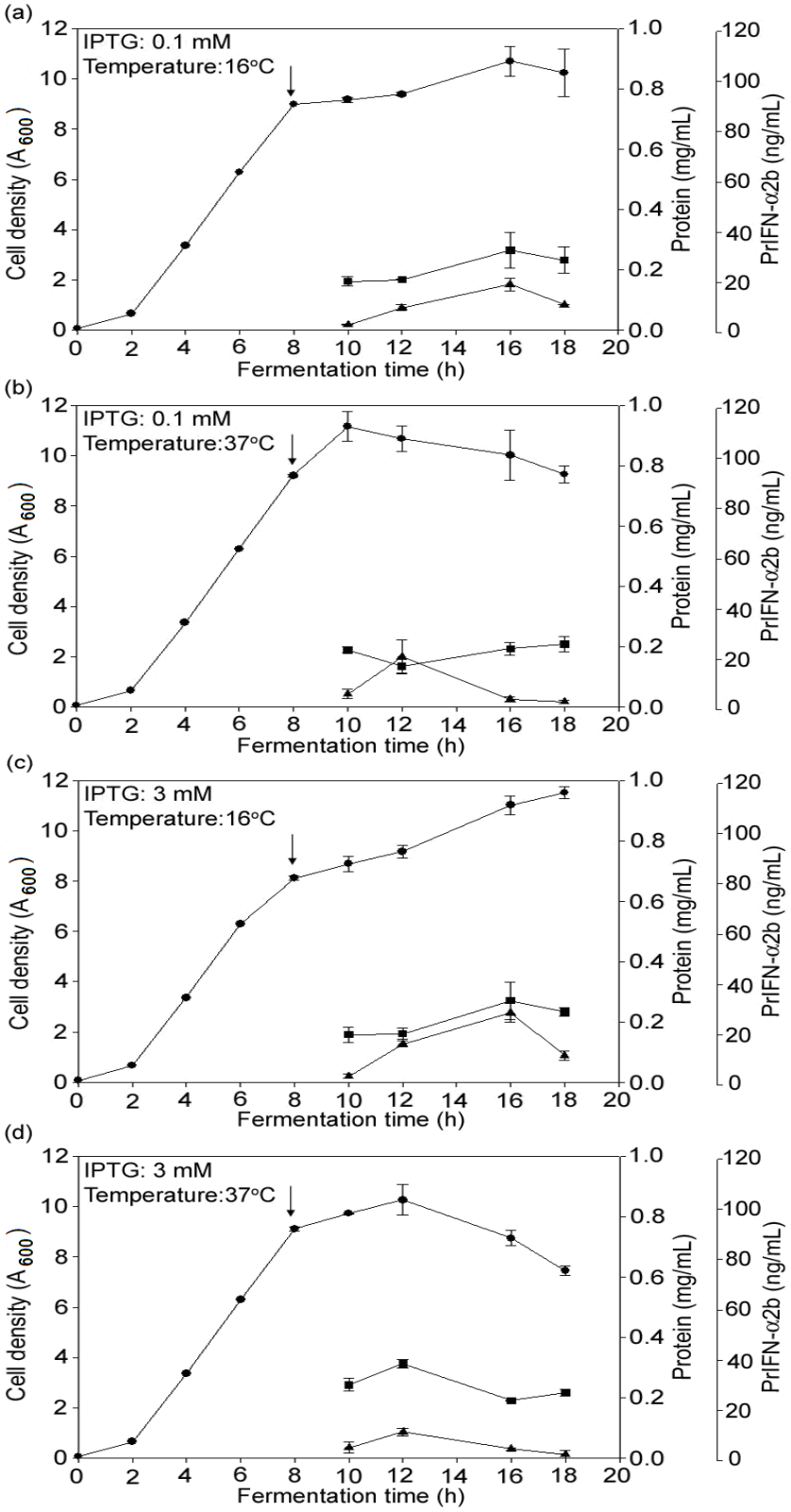

Figure 5. Growth profile of *E. coli* and production of PrIFN-α2b and periplasmic protein during fermentation when induced at late log phase with different IPTG concentrations and induction temperatures. [●] Cell density; [■] protein; [▲] PrIFN-α2b. The error bar represents the standard deviation and the arrow represents induction starting point.

accumulation ofacetic acid. In addition, induction at a lower specific growth rate would make carbon (energy) to be a limiting factor. Similar findings were also obtained in this study. Additionally, high energy is required for the translocation process of proteins into periplasm. Therefore, induction range between early-log phase and middle-log are preferred for improvement of PrIFN-α2b production.

The temperature during induction also plays a significant role in determining the maximum product yield in periplasmic *E. coli*. It is also a major factor that influences the activity of enzymes inside the cells and the solubility of protein produced. In fact, maximal activity of the *lac* and *tac* promoters occurs at temperatures between 37 and 39°C (Donovan et al., 1996; Bronikowski et al., 2001). It is not surprising that at this cultivation temperature, *E. coli* produced higher recombinant protein production as well as protein itself. However, the effect is different for periplasmic expression (Mergulhao et al., 2004; Mergulhao et al., 2005; Mergulhao and Monteiro, 2007). When more proteins were produced, the transport machinery of *E. coli* seems to be limited. Hence, the rate of transfer of premature protein was lowered (Mergulhao et al., 2004; Mergulhao and Monteiro, 2007; Balagurunathan and Jayaraman, 2008). In this case, high expression rate which was driven by high induction strength was not favorable for periplasmic expression. Hence, lower temperature that facilitates soluble protein production was favorable to enhance the rate of protein transfer across the membrane (Gasser et al., 2008).

At lower temperature (e.g. 16°C) the metabolism of cells is reduced, followed by inhibition of enzyme activity responsible for protein expression. In addition, the biological function of the membrane would be lost as the temperature decreases. Membranes are essentially colloidal solutions of phospholipids and protein in a fluid phase and it is only in this fluid phase that they are biologically functional. When the temperature decreases, membranes become increasingly viscous with decreasing membrane fluidity. Below certain temperature, these membranes change to a gel (solid) phase when biological function is lost (Nedwell, 1999). As a consequence, the synthesis rates of enzymes belonging to the biosynthetic machinery (e.g., RNA polymerase enzyme) and translational factors decreased more strongly in the culture synthesizing the recombinant protein (Hoffmann et al., 2004).

High level expression using strong promoter like *tac* and T7 systems require a short induction period (Sandén et al., 2003). Longer induction period are applied when the level of expression is low and the availability of nutrients is high. In some cases, the production might be reduced to zero and at worst, the protein product is degraded. Furthermore, reduction of growth could also be seen when the nutrients became exhausted (Chen and Morgan, 2006). Increase in the time for induction process usually has relative effect on the protein leakage from periplasmic space into the culture medium (Bäcklund et al., 2008) and this leakage is partly associated with autocell lysis (Somerville et al., 1994). In our case, the length of induction was based on the fermentation condition employed. Longer induction time showed higher PrIFN-α2b production when low level of induction strength was introduced. On the other hand, lower PrIFN-α2b production was observed when high expression strength was introduced. Besides, the time of induction (induction point) also played a significant role in determining the duration of induction time. Hence the length of induction could be determined once the other conditions were chosen for higher expression.

Conclusion

Results from this study have demonstrated that the induction strategy greatly influenced the *E. coli* fermentation for enhancement of PrIFN-α2b production. Lower strength of induction, narrow range near the middle level of induction point and temperature was preferred to maximize the PrIFN-α2b production by the recombinant *E. coli*. Length of induction could be determined based on the future optimized condition. Besides, further optimization of these factors using response surface methodology to enhance the PrIFN-α2b production in recombinant *E. coli* RG2 (DE3) is being carried out in our laboratory.

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Full Length Research Paper

Optimization, economization and characterization of cellulase produced by marine *Streptomyces ruber*

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Cellulase is a very important enzyme due to its great industrial applications. Six marine strains of actinomycetes were screened for their carboxymethyl cellulase (CMCase) productivity. *Streptomyces ruber* was chosen to be the best producing strain. The highest enzyme production (25.6 U/ml) was detected at pH 6 and 40°C after 7 days of incubation. Plackett-Burman design was applied to optimize the different culture conditions affecting enzyme production. Results showed that a high concentration of KH_2PO_4, and a low concentration of $MgSO_4$ had a significant effect on enzyme production. Rice straw was used as a low cost source of cellulose. It was found that 30 g/l rice straw was the suitable concentration for maximum enzyme production. Partial purification of cellulase enzyme using an anion-exchange chromatography resulted in the detection of two different types of CMCases, type I and II, with specific activity of 4239.697 and 846.752 U/mg, respectively. Moreover, estimation of their molecular weight revealed 27.0 kDa for cellulase type I and 24.0 kDa for cellulase type II. It could be concluded that *S. ruber* is a powerful cellulase producer strain under our tested experimental conditions.

Key words: Cellulase production, *Streptomyces ruber,* Plackett-Burman design, rice straw, enzyme characterization.

INTRODUCTION

Cellulases have attracted much interest because of the diversity of their applications. The major industrial applications of cellulases are in textile industry for 'biopolishing' of fabrics and for producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness. Moreover, they are used in animal feeds for improving nutritional quality and digestibility, in processing of fruit juices, and in baking, while de-inking of paper is yet another emerging application. A potential challenging area where cellulases would have a central role is the bioconversion of renewable cellulosic biomass to commodity chemicals (Ibrahim and El-Diwany, 2007).

Actinomycetes, one of the known cellulase producers, has attracted considerable research interest due to its potential application in the recovery of fermentable sugars from cellulose that can be of benefit to human consumption and to the ease of their growth (Jang and Chen, 2003; Arunachalam et al., 2010). Streptomycetes are the largest and well-studied group of actinomycetes. A wide variety of bacteria are known for their production of hydrolytic enzymes with streptomycetes being the best known enzyme producers (Chellapandi and Jani, 2008).

The optimization of fermentation conditions is an important problem in the development of economically feasible bioprocesses. Combinatorial interactions of medium

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Abbreviations: CMC, Carboxymethyl cellulose; **CMCase,** carboxymethyl-cellulase; **DNS,** dinitrosalicylic acid; **DEAE,** diethylaminoethyl.

components with the production of the desired compounds are numerous and optimum processes may be developed using an effective experimental design procedures (Hao et al., 2006; El-Sersy et al., 2010).

Rice cultivation produces large quantities of straw, as an agricultural waste, ranging from 2 to about 9 tons/ha globally. Components of rice straw are mainly cellulose and hemicellulose encrusted by lignin, in addition to a small amount of protein, which makes it high in C:N ratio (Abdulla, 2007). Products of agricultural practices result in the generation of numerous structural plant components which contribute to environmental pollution problems. One of the easiest ways of handling pollution problems is by the re-use of these agricultural wastes in the production of highly important useful enzymes (Odeniyi et al., 2009a).

In the present study, we aim to study the optimization and economization of cellulase production using marine *Streptomyces ruber* from a low-cost source. Moreover the study extended to investigate the characterization of the enzyme.

MATERIALS AND METHODS

Microorganisms

Six actinomycetes isolates used in the current investigation were kindly provided by Dr. Gehan Abou Elela (Associate professor of Marine Microbiology, National Institute of Oceanography and Fisheries, Alexandria - Egypt). Isolates were recovered from marine sediments in a previous study, They were tentatively identified as *Streptoverticillium morookaense, Streptomyces globosus, Streptomyces alanosinicus, S. ruber, Streptomyces gancidicus and Nocardiopsis aegyptia,* following Bergy's Manual of Systematic Bacteriology (Holt and Williams, 1994) by Al-Azhar University Fermentation Biotechnology and Applied Microbiology (Ferm. BAM) Center, Egypt.

Electron microscopy studies

Electron microscopy was performed using the cover slip technique. The cover slip was cut with a glass file and a suitable fragment with growth on it was chosen. It was mounted on a specimen-tube, coated with gold-palladium under vacuum and examined with a scanning electron microscope (Joel ISM-5300) operating at 10 KV.

Screening for cellulases producing actinomycetes strains

A preliminary analysis for cellulolytic activity was conducted using Congo red dye. All strains were grown on carboxymethyl cellulose (CMC) agar containing (g/l) KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; $FeSO_4.7H_2O$, 0.01; $MnSO_4.H_2O$, 0.01; NH_4NO_3, 0.3; (CMC), 10.0 and agar 12.0. The pH was adjusted to 7.0 with 1.0 M NaOH. The CMC agar plates were incubated at 30°C for 7 days to allow for the secretion of cellulase. At the end of the incubation, the agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 min. The Congo red solution was then poured off, and the plates were further treated by flooding with 1.0 M NaCl for 15 min. The formation of a clear zone of hydrolysis indicated cellulose degradation. The ratio of the clear zone diameter to colony diameter was measured in order to select the highest cellulase activity producer. The largest ratio was assumed to contain the highest activity (Ariffin et al., 2006).

Production of crude cellulases

The production of crude enzyme was carried out in the same medium used for screening without the addition of agar. A loop full of culture from agar plates was inoculated into glass tubes containing 5 ml of production medium, and incubated at 120 rpm and 30°C. This culture was then inoculated ($9x10^4$ CFU/ml) into a 250-ml capacity Erlenmeyer flask containing 100 ml of the same medium, 2 ml aliquots were withdrawn and were centrifuged at 10,000 g for 10 min. Cellulase activities were measured in cell-free supernatant which was used as the source of crude cellulase enzymes.

Carboxymethyl-cellulase (CMCase) activity

CMCase activity was assayed using a modified method described by Wood and Bhat (1998) with some modifications. 0.2 ml of culture filtrate was added to 1.8 ml of 1% CMC prepared in 0.05M sodium citrate buffer (pH 4.8) in a test tube and incubated at 40°C for 30 min. The reaction was terminated by adding 3.0 ml of dinitrosalicylic acid (DNS) reagent and by subsequently placing the reagent tubes in a water bath at 100°C for 15 min. 1 ml of Rochelle salt solution (40 g Rochelle salt in distilled water to make the volume 100 ml) was then added to stabilize the colour. Using spectrophotometer, absorbance was recorded at 575 nm against the blank (0.05 M sodium citrate buffer). One unit of CMCase activity was expressed as 1.0 µM of glucose liberated per ml enzyme per min (Ariffin et al., 2006). By using a calibration curve for glucose, one unit of enzyme activity was defined as the amount of enzyme that released 1 µM of glucose per min.

Reducing sugars content

Reducing sugars analysis was conducted by using 2 ml of sample which was added to 3 ml of DNS and boiled for 15 min. After boiling, 1 ml of Rochelle salt was added. The absorbance was recorded at 575 nm using a spectrophotometer against the blank of distilled water (Ariffin et al., 2006).

Protein determination

Protein content was determined by the assay method which is based on the method of Bradford (1976). In this assay, 5 ml dye reagent was pipetted into 100 µl of sample solution. The mixture was then incubated at room temperature for at least 5 min, but not more than 60 min. The absorbance was measured at 280 nm against the blank of deionized water.

Biomass yield

Culture broth was centrifuged and the cell pellet was washed twice with phosphate buffer (0.1 M, pH 7). The cell mass was dried at 80°C to constant weight (Bidlan et al., 2007).

Effect of temperature and pH on cellulase production

The influence of incubating temperature on cellulase production was

Table 1. Screening test of different actinomycetes strains grown on carboxymethyl cellulose (CMC) and their clearance zones (mm) after 7 days of incubation.

Strain	Diameter of the colony (mm)	Diameter of clearance zone (mm)
Streptoverticillium morookaense	6	10
Streptomyces globosus	5	10
Streptomyces alanosinicus	7	15
Streptomyces ruber	9	25
Streptomyces gancidicus	7	15
Nocardiopsis aegyptia	5	10

determined by measuring enzyme activity at temperatures ranging from 30 to 60°C under standard assay conditions. The effect of pH-value on enzyme production was determined by measuring the enzyme activity at different pH-values ranging from 5 to 9.

Experimental design and optimization

Plackett-Burman is a technique devoted to the screening of controlled experimental factors and the measurement of their responses, according to one or more selected criteria. A prior knowledge and understanding of the process and the process variables under investigation are necessary for achieving more realistic results. Plackett-Burman design was used to pick factors that influence cellulase production significantly and insignificant ones were eliminated in order to obtain smaller, more manageable set of factors (Hao et al., 2006).

For each variable, a high (+) and low (-) levels were tested. The examined variables in this experiment and their levels are shown in Table 2. Eight different trials were performed in duplicates. Rows in Table 3 represent the different trials (row number 9 represents the basal control). The main effect of each variable was determined with the following equation:

$$Ex_i = (Mi^+ - Mi^-) / N$$

Where, Ex_i is the variable main effect, and Mi^+, Mi^- are the enzyme produce (units) in the trials, where the independent variable was present in high and low concentrations, respectively, and N is the number of trials divided by two. Statistical t-values for equal unpaired samples were calculated using Microsoft Excel to determine the variable significance. From main effect results, an optimized medium was predicted which will give maximum enzyme productivity.

Verification experiment

A verification experiment was carried out in duplicates. The predicted optimum levels of the independent variables were examined and compared to the basal condition setting and the average of enzyme production was calculated.

Cellulase production using agro-industrial residues

Rice straw was provided from a rice field at Kafr El-Dawar City. It was dried at 50°C then milled well several times until it gave a powdery appearance. Optimized medium was prepared without carbon and nitrogen sources but supplemented with different concentrations (10, 15, 20, 25, 30 and 40 g) of rice straw. All flasks were incubated and enzyme activity was assayed.

Partial purification of cellulase enzyme and polyacrylamide gel electrophoresis (PAGE)

Partial purification of cellulase enzyme produced from the growth of *S. ruber* on rice straw under optimized conditions, was performed using KTA Fast Protein Liquid Chromatography (FPLC) (Amersham Pharmacia Biotec. The diethylaminoethyl cellulose (DEAE)-sepharose CL 6B column as an anion-exchange liquid chromatography technique was used for separation and purification of cellulase enzyme, while the running buffer was 20 mM sodium acetate (pH 6) and the elution buffer was 1.0 M NaCl in 20 mM sodium acetate (pH 6). The elution rate was 1.0 ml/min in a sample volume of 5 ml.

The purified cellulase was desalted and concentrated using the ultrafiltration tubes at a speed of 3,000 rpm for 30 min at 4°C in Centricon 10 (Amicon, USA) ultrafiltration concentrators (membrane cut off of 10 kDa).

For the detection of the molecular weight and cellulase enzyme homogeneity, the denaturing sodium dodecylsulphate-PAGE was used (Laemmli, 1970), on SCIE-PLAS TV100 YK-EBSYS TV-Modular Electroblotting System with 10% polyacrylamide. Fermentas protein was used as a molecular weight marker.

RESULTS

Screening of cellulase producers

Screening of actinomycetes was conducted using the Congo red test as a preliminary study for choosing the best cellulase producers. After 7 days of incubation, all strains of actinomycetes showed signs of growth on CMC agar and demonstrated positive results in the Congo red test. Table 1 shows that *S. ruber* gave the highest ratio of clear zone diameter to colony diameter. This indicated more cellulose degradation in CMC agar plates cultured with *S. ruber* as compared to plates cultured with other strains. Growth of *S. ruber* after 7 days at 30°C was illustrated by scanning electron micrograph (Figure 1).

Effect of temperature and pH-value on enzyme production

Using *S. ruber* as a cellulase producer, the effect of temperature on the production of crude cellulases was determined at various temperatures ranging from 30 to

Table 2. Independent variables affecting cellulase production and their levels in the Plackett-Burman design.

Factor (g/l)	Symbol	Level		
		-1	0	1
KH_2PO_4	KH_2	0.5	1	1.5
$MgSO_4$	Mg	0.1	0.5	1.5
NaCl	Na	0.0	0.5	1.5
$FeSO_4$	Fe	0.0	0.01	0.05
$MnSO_4$	Mn	0.0	0.01	0.05
NH_4NO_3	NH	0.1	0.3	0.5
Inoculum size (ml)	IS	0.5	1	1.5

Table 3. The applied Plackett-Burman experimental design for seven cultural variables and their enzyme activity results.

Trials	KH_2	Mg	Na	Fe	Mn	NH	IS	Enzyme activity (U/ml)	Biomass (mg/ml)	Reducing sugar (µg/ml)	Protein content (mg/ml)	Specific activity (U/mg)
1	-1	-1	-1	1	1	1	-1	27.7	5.79	257.14	0.39	71.03
2	1	-1	-1	-1	-1	1	1	35.6	6.1	300	0.52	68.46
3	-1	1	-1	-1	1	-1	1	17.9	0.697	120	0.22	81.36
4	1	1	-1	1	-1	-1	-1	19.4	3.61	197.1	0.25	77.6
5	-1	-1	1	1	-1	-1	1	18.4	0.92	177.1	0.23	80
6	1	-1	1	-1	1	-1	-1	39.5	11.12	385	0.62	63.71
7	-1	1	1	-1	-1	1	-1	18.3	3.32	187.1	0.21	87.14
8	1	1	1	1	1	1	1	24.7	3.65	202.8	0.33	74.85
9	0	0	0	0	0	0	0	25.6	5.31	218	0.32	80

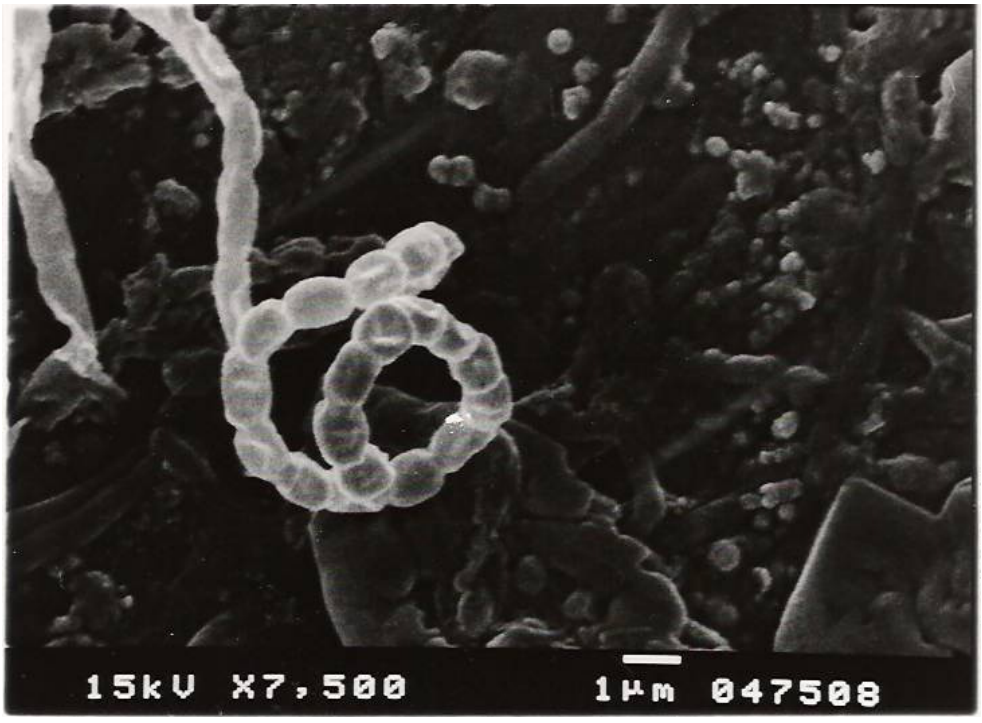

Figure 1. Scanning electron micrograph showing the growth of *S. ruber* after 7 days at 30°C.

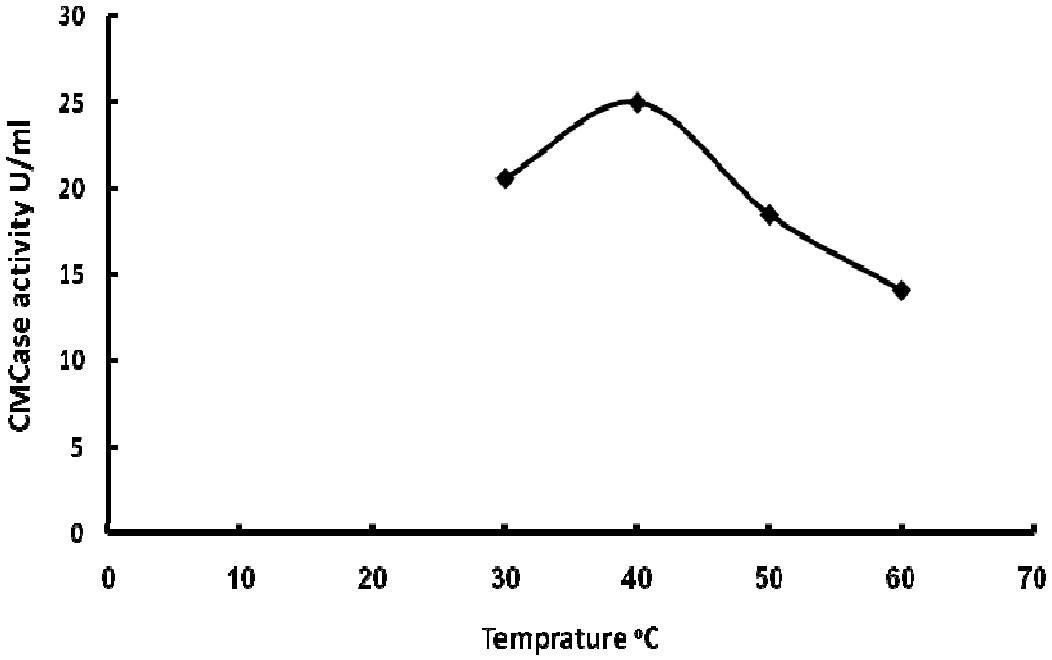

Figure 2. Effect of temperature on the cellulase enzyme activity produced from *S. ruber*.

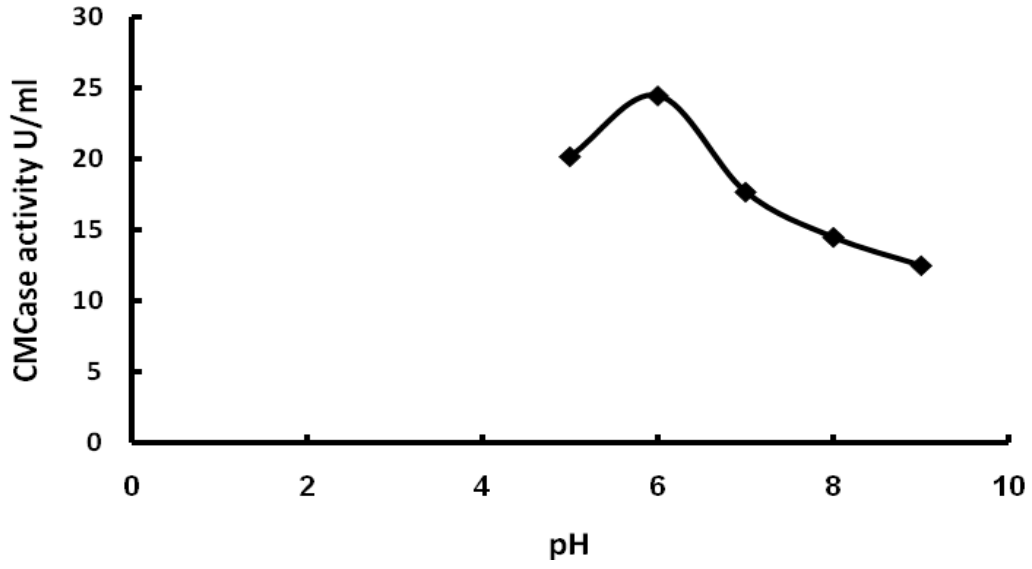

Figure 3. Effect of pH-value on the cellulase enzyme activity produced from *S. ruber*.

60°C at pH 7 (Figure 2). The enzyme showed a good production between 35 to 45°C with maximum activity at 40°C. The effect of pH-value on cellulase enzyme activity produced from *S. ruber* was examined at various pH values ranging from 5.0 to 9.0 as shown in Figure 3. The enzyme shows high activity at a broad range of pH values (pH 5.5 - 7) with optimal pH at 6.0. The enzyme production had about 50% decrease at pH 9.

Evaluation of different parameters affecting cellulase production

Plackett-Burman design has been employed to evaluate the significant effect of the seven different culture elements on the production of cellulase using a basal medium. The main effect of each constituent on the cellulase production (Table 4) was calculated as the difference between

Table 4. Statistical analysis for the results applied to the Plackett-Burman experimental design.

Variable	Enzyme activity (U/ml)		Biomass (mg/ml)	
	Main effect	*t*-value*	Main effect	*t*-value
KH_2PO_4	9.22	1.8	3.44	-4.13
$MgSO_4$ (g/l)	-10.2	-2.1	-3.18	-3.99
NaCl ((g/l)	0.05	0.01	0.7	-3.47
$FeSO_4$ ((g/l)	-5.2	-0.9	-1.81	-3.6
$MnSO_4$	4.6	0.7	1.82	-3.61
NH_4NO_3	2.8	0.4	0.635	-3.47
Inoculum size (ml)	-2.1	-0.3	-3.12	-3.98

*t-value significant at the 1% level = 3.70; 5% level = 2.45; 10% level = 1.94; 20% level = 1.37.
Standard t-values were obtained from statistical methods (Cochran and Snedecor, 1989).

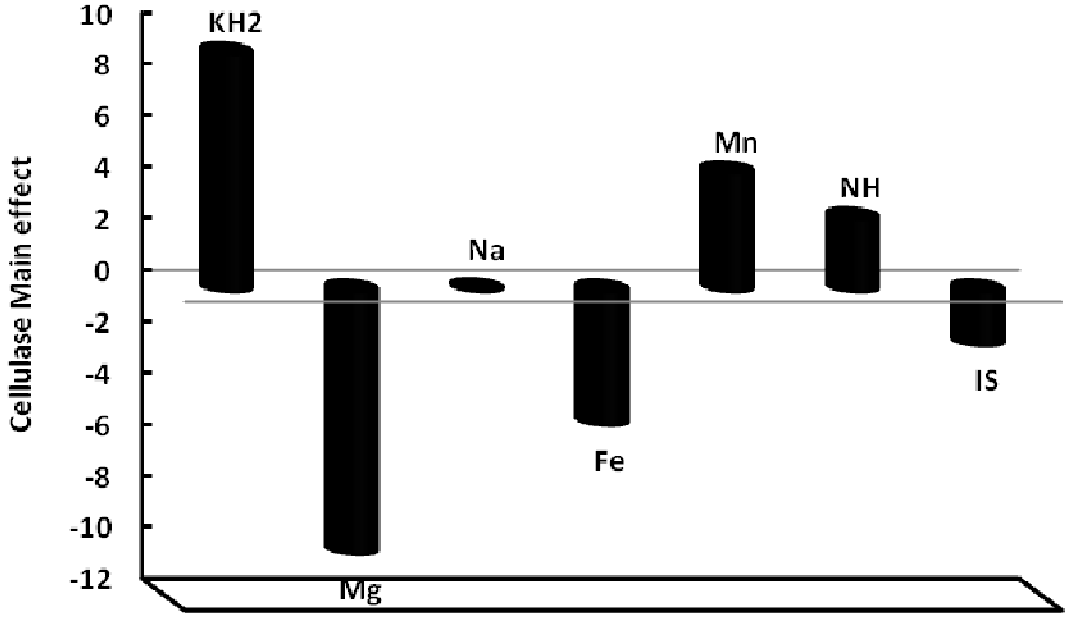

Figure 4. Enzyme production main effect of the medium constituents after applying Plackett-Burman experimental design.

the average response measurement calculated at the higher (+) and lower (-) levels of the constituent (Table 3). Main effect results (Figure 4) showed that KH_2PO_4 had a highly positive main effect which positively affected the enzyme produce. Also, $MnSO_4$ and NH_4NO_3 had positive main effect. On the other hand, $MgSO_4$, $FeSO_4$ and inoculum size showed considerable high negative main effect on enzyme production. Moreover, main effect results were confirmed by calculating t-test. KH_2PO_4, and $MgSO_4$ showed significant effect on enzyme production (Table 4). Figure 5 illustrates the interaction between KH_2PO_4, and $MgSO_4$ concentrations (g/l) on the cellulase production. We can notice from this figure that the high concentration of KH_2PO_4 with the low concentration of $MgSO_4$ increased the cellulase production. Finally, it can be concluded that the optimum formula of cellulase production medium is as follows (g/l): KH_2PO_4, 1.5; $MgSO_4$, 0.1; $MnSO_4$, 0.05; NH_4NO_3, 0.5; NaCl, 1.5 with inoculum size of 0.5 ml.

Verification experiment

A duplicate of experiment was performed to verify the optimization result in order to validate the developed optimized medium. The optimized medium recorded a higher enzymatic activity (40 U/ml) than that of the basal by 1.6 fold increase. These results confirm the validity of

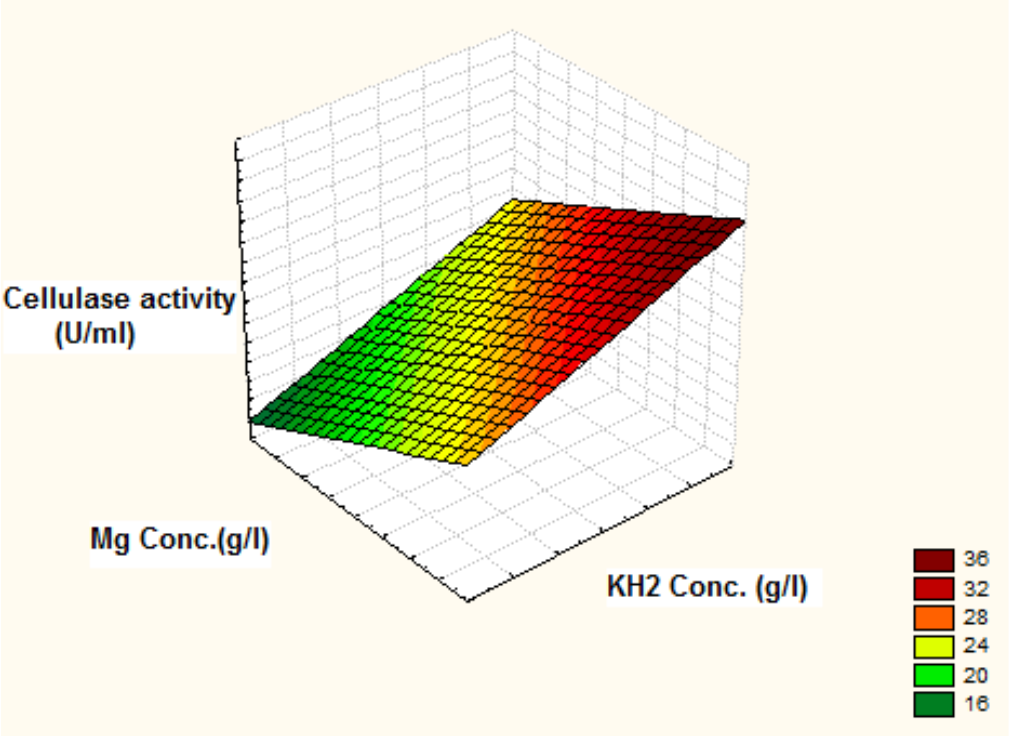

Figure 5. Interaction between KH_2PO_4, and $MgSO_4$ concentrations (g/l) on the activity of the cellulase enzyme (U/ml).

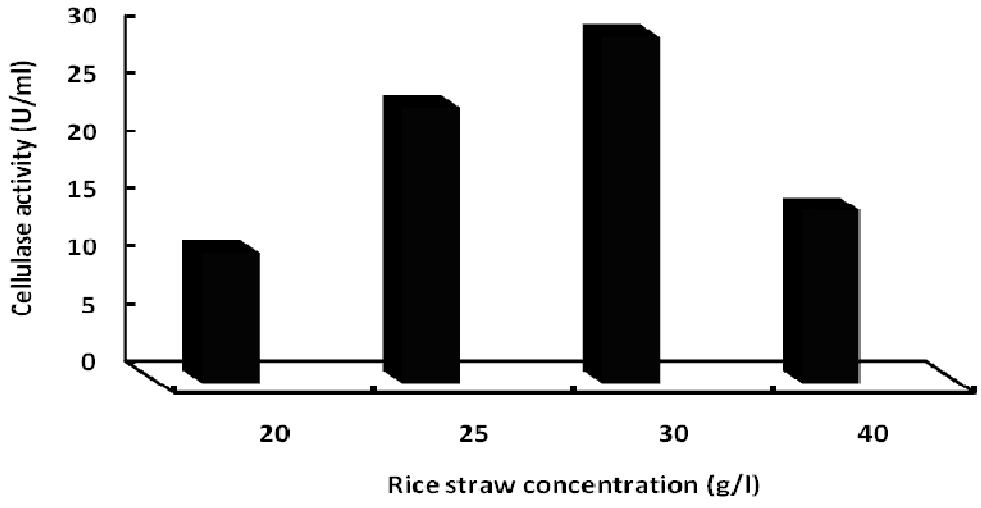

Figure 6. Effect of using rice straw at different concentrations on cellulase enzyme activities.

the optimized medium.

Effect of using rice straw at different concentrations on cellulase enzyme activity

This experiment was done for the determination of the optimum rice straw concentration which can be used for giving rise to a maximum cellulase production. Different concentrations of rice straw were used as illustrated in Figure 6. Results revealed that a concentration of 30 g/l rice straw could give a high cellulase enzyme activity (30 U/ml) while increasing rice straw concentration to 40 g/l, dramatically decreases the enzyme activity (15 U/ml) to

Table 5. Results of partially purified cellulase enzyme.

Cellulase types	Protein content (mg/ml)	Cellulase activity (U/ml)	Specific activity (U/mg)
Total Cellulase in the culture filtrate	1.55	659.25	425.32
Cellulase type I	0.099	419.730	4239.697
Cellulase type II	0.342	289.415	846.752

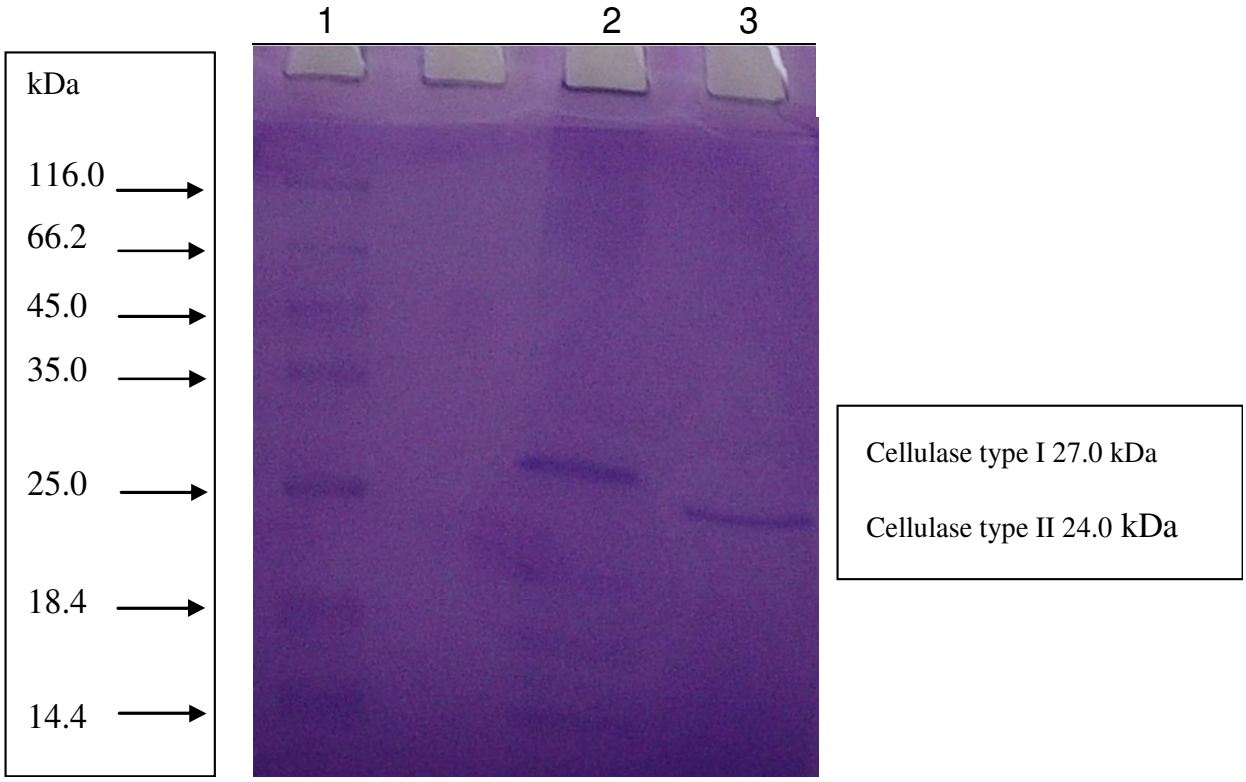

Figure 7. SDS-gel polyacrylamide electrophoresis (SDS-PAGE) showing the partially purified CMCases. Lane 1: Fermentas protein standard (cat. No. SM0431) with range from 14.4 - 116.0 kDa; lane 2: partially purified cellulase enzyme type I; lane 3: partially purified cellulase enzyme type II.

50%.

Partial purification of cellulase and polyacrylamide gel electrophoresis

The enzyme was partially purified using the DEAE-sepharose CL 6B as an anion-exchange chromatography and the fractions were tested for the CMCases. This resulted in the appearance of two different type of CMCases, type I and type II. The corresponding fractions of each type was pooled, dialyzed and tested for protein contents and cellulase activity (Table 5).

Comparing their specific activities with the specific activity of the culture filtrate indicated that the anion-exchange chromatography as a separation technique was an excellent method for cellulase purification, since the purification-fold for cellulase type I was found to be almost 10 times while purification-fold for cellulase type II was found to be almost two times.

SDS-PAGE with Coomassie-brilliant blue staining was conducted to determine the molecular weight of the two types of cellulases. The estimated molecular weight of cellulase type I was 27.0 kDa, while for cellulase type II, it was 24.0 kDa (Figure 7).

DISCUSSION

Streptomyces species have always been a source of thousands of bioactive compounds. Enzymes are one of the important products of this unusual group of bacteria. *Streptomycetes* sp. with potential cellulolytic activity is subjected to produce endoglucanase in liquid culture

(Chellapandi and Jani, 2008). Most of the *Streptomyces* isolates recovered from the different soils of Jordan produced fiber hydrolytic enzymes. Cellulase, which is considered one of the most important hydrolytic enzymes, was produced by most of the isolates (94%) (Jaradat et al., 2008; Arunachalam et al., 2010) studied in cellulose producing actinomycetes from soil of Southern-West Ghats, Tamilandu, India. Screening of actinomycetes strains was conducted by using the Congo red test. Since the sole carbon source in CMC agar was cellulose, the result of the test was strong evidence that cellulase was produced.

S. ruber gave the highest ratio of clear zone diameter to colony diameter. This indicated more cellulose degradation in CMC agar plate cultured with *S. ruber* as compared to plates cultured with the other strains.

Temperature and pH-values were found to be important parameters that influenced enzyme activities and production (Odeniyi et al., 2009b). CMCase enzyme from *S. ruber* was found active over a pH range of 5.5 - 7 with maximum activity at pH 6. Theberge et al. (1992) showed that the optimum pH for endoglucanase from a strain of *Streptomyces lividans* was 5.5. Jaradat et al. (2008) also found that CMCase enzyme from *Streptomyces sp*. (strain J2) was active over a pH range of 4 - 7 with maximum activity at pH 6 . Ariffin et al. (2006) reported that maximum activity of CMCase from *Bacillus pumilus* was detected at pH 6. However, Solingen et al. (2001) studied the alkaline novel *Streptomyces* species isolated from east African soda lakes which showed an optimal pH of 8.

The maximum CMCase activity of *S. rubber* was recorded at 40°C and the optimum range, 35 - 45°C. These results are similar to that reported by Alam et al. (2004) who studied the heavy growth and high cellulase activity by *Streptomyces omiyaensis* at 35 and 40°C. Furthermore, McCarthy (1987) reported an optimal temperature for cellulase activity in the range of 40 - 55°C for several *Streptomyces* species including *Streptomyces lividans, Streptomyces flavogrisus*, and *Streptomyces nitrosporus*. On the other hand, higher temperatures were optimum for CMCase production. Jaradat et al. (2008) found that the maximum CMCase activity of *Streptomyces sp.* (isolate J2) was recorded at 60°C with no significant difference ($p < 0.05$) between 50 and 60°C. In addition, Jang and Chen (2003) described a CMCase produced by a *Streptomyces* T3-1 with an optimum temperature of 50°C whereas Schrempf and Walter (1995) described a CMCase production by a *Streptomyces reticuli* at an optimum temperature of 55°C.

Prolonged incubation periods (7 days) were required to obtain maximum enzymatic production by streptomycetes and that agrees with Alam et al. (2004) and Arunachalam et al. (2010).

Statistical experimental designs are powerful tools for the rapid search of key factors from a multivariable system and minimizing the error in determining the effect of parameters and the results are achieved in an economical manner (El-Sersy, 2007; Abou-Elela et al., 2009). One of the advantages of the Plackett-Burman design is that it helps to rank the effect of different variables on the measured response independent of its nature (either nutritional or physical factor) or sign (whether contributes positively or negatively) (Youssef and Berekaa, 2009).

Youssef and Berekaa (2009) studied the production of endoglucanase by *Aspergillus terreus* by applying the Plackett-Burman design for optimization of process parameters and this study agreed with our results in that, both KH_2SO_4 and $MgSO_4$ positively affected CMCase production. High levels of KH_2SO_4 and low levels of $MgSO_4$, maximize enzyme production. Hao et al. (2006) studied optimization of the medium for the production of cellulase by the mutant *Trichoderma reesei* WX-112 using response surface methodology and the results showed that the presence of K_2HPO_4 positively affected cellulase production. In our study, the predicted medium for optimum production is as follows: KH_2SO_4, 1.5; NaCl, 1.5; $MnSO_4$, 0.05; NH_4NO_3, 0.5; $MgSO_4$, 0.1 and inoculum size, 0.5 ml. Moreover, verification experiment using this medium, increased cellulase activity by 1.6 folds.

Rice cultivation produces large quantities of straw as an agriculture waste. Components of rice straw are mainly cellulose and hemicellulose encrusted by lignin, in addition to a small amount of protein. It is resistant to microbial decomposition compared to straw from other protein-rich grains such as wheat and barley (Parr et al., 1992). Egypt is the largest rice producer in the Near East region (Sabaa and Sharaf, 2000). Currently, the major practice to eliminate such massive amounts of post-harvest rice residues is field open air burning. The produced black smoke represents a threat to public health; it also introduces carbon monoxide and some nitrogen dioxide, which has statistically significant effect on asthma morbidity (Schwartz et al., 1993). Low cost production of cellulases from different wastes had been studied by many workers. Shabeb et al. (2010) studied the low cost production of cellulase from molasses by *B. subtilis* KO and the economic value of the product. Immanuel et al. (2007) reported that *Aspergillus niger* and *Aspergillus fumigatus* were capable of producing cellulase enzyme optimally at 40 and 50°C during growth on coir waste and sawdust, respectively. In this study, microbial decomposition by exhausting rice straw (as pollutant) and the economic production of cellulase were valuable aims to environmental protection and industrial progress.

Partial purification of cellulase was conducted by Bajaj et al. (2009) where the endoglucanase was purified to the extent of 9.06 folds by salt precipitation and DEAE-cellulose chromatography. Moreover, the study was extended to investigate endoglucanase molecular weight which was approximately 54 kDa as examined by SDS-PAGE. In our study, partial purification of cellulase using

anion-exchange chromatography resulted in two types of cellulases, type I and II. Cellulase type I was purified to the extent of 10 fold while type II was about 2 fold. Molecular weight estimation using SDS-PAGE showed that the estimated molecular weight of cellulase type I was 27.0 kDa while for cellulase type II, it was 24.0 kDa. Conclusively, to the best of our knowledge, this is the first report on the high level of production of cellulase by *S. ruber*, from rice straw.

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Full Length Research Paper

The antibacterial potential of the seaweeds (*Rhodophyceae*) of the Strait of Gibraltar and the Mediterranean Coast of Morocco

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The antibacterial activity of extracts from 26 marine *Rhodophyceae* (8 Ceramiales, 7 Gelidiales, 9 Gigartinales, 1 Bonnemaisoniales and 1 Rhodymeniales) was studied to assess their potential in the pharmaceutical industry. Their bioactivity was analysed from crude methanolic extracts of dried samples against three gram-positive bacteria and two gram-negative bacteria using the disc diffusion technique. The samples were collected from Gibraltar and the Moroccan Mediterranean coast. Of the macroalgae analysed, 96% of extracts were active against at least one of the five test microorganisms. *Staphylococcus aureus* was the most susceptible microorganism. Methanolic extracts of all seaweed extracts tested in the present study exhibited a broad spectrum of antibacterial activity with inhibition diameters ranging from 10 to 35 mm. An extract of *Hypnea musciformis* exhibited high antibacterial activity against all the bacteria tested. The results of the present study confirmed the potential use of seaweed extracts as a source of antibacterial compounds.

Key words: Antibacterial activity, methanolic extracts, pathogenic bacteria, *Rhodophyceae*.

INTRODUCTION

Several macroalgae produce bioactive metabolites in response to ecological pressures such as competition for space, deterrence of predation and the ability to reproduce (König et al., 1994). In a marine environment, where all surfaces are constantly exposed to the threat of surface colonisation, sessile organisms remain relatively free from biofouling. These sedentary organisms control epibionts in particular marine bacteria by effective antifouling mechanisms (Hellio et al. 2001 and Bazes et al. 2006).

Macroalgae are a rich source of natural bioactive products although little has been done to define an ecological role for these compounds (Ballantine et al., 1987; De Nys et al., 1995; Shanmugam and Mody, 2000; Suzuki et al., 2001; Vairappan et al., 2001b). They may, therefore possess chemical defences to prevent the colonisation of their surface. The use of marine natural products capable of inhibiting bacteria development offers rich pharmacological potential (Kornprobst, 2005). Numerous reports show macroalgae to present a broad range of biological activities such as antibacterial (Fenical and Paul, 1984; Mtolera and Semesi, 1996; Gonzalez et al., 2001; Selvin and Lipton, 2004; Karabay-yavasoglu et al., 2007; Salvador et al., 2007), antifungal (Moreau et al., 1984; Tariq, 1991; Mayer, 2002; Mayer et al., 2007, 2009) and antiviral (Bourgougnon et al., 1993, 1994; Hudson et al., 1999; Serkedjieva 2000, 2003; Mayer, 2002; Ghosh et al., 2004; Zandi et al., 2007) activities.

The ability of seaweeds to produce secondary metabolites of antimicrobial value, such as volatile components (phenols, terpenes) (Ozdemir et al., 2004; König et al., 1999a; Awad, 2004; Glombitza et al., 1985; Xu et al., 2003, Karabay-yavasoglu et al., 2007; Vairappan et al., 2001a,b; Vairappan, 2003), steroids (Awad, 2000), phlorotannins (Nagayama et al., 2002) and lipids (Ballantine et al., 1987; Rossel and Srivastava, 1987; Freile-Pelegrini and Morales, 2004) has already been studied.

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In contrast to the brown and green algae, the red algae are more known to produce halogenated metabolites, particularly bromine and iodine (McConnell and Fenical, 1977; Fenical, 1981; König et al., 1999b). The orders of Nemaliales, Gigartinales, Ceramiales, Rhodymeniales and Cryptonemiales have been shown to be engaged in biological halogenations yielding a diverse array of organic compounds (McConnell and Fenical, 1977).

The Moroccan Coast is particularly rich in algal bio-diversity and constitutes a reserve of species of considerable economic, social and ecologic potential. Today however, only the *Gelidium sesquipedale* species is exploited in Morocco. Between 1995 and 2000, the extraction of agar-agar increased by 7968 at 12.068 tons per year. If other horizons could be prospected and other algae developed, the pressure on the traditional species could decrease. Nevertheless, little is known about the antimicrobial activity of algae from the coast of Morocco, with the exception of some studies carried out on the Atlantic coast and reported in the work of Abourriche et al. (1999), Etahiri et al. (2003), Moujahidi et al. (2004) and Souhaili et al. (2004).

The purpose of our work is to research the new antibiotic compounds to be extracted from benthic extracts of *Rhodophyceae* of the Mediterranean Coast of Morocco for a possible valorisation and exploitation in a spirit of sustainable development. In the present investi-gation, a successful attempt was made to determine the antibacterial activity of methanolic extracts from marine algae belonging to five orders: Ceramiales, Gelidiales, Gigartinales, Rhodymeniales and Bonnemaisoniales against five pathogenic terrestrial bacteria.

MATERIALS AND METHODS

The collection of seaweeds

Seaweeds were collected by hand using Scuba diving or snorkelling (1-4m depth) and preserved on ice until further processing. Twenty-six were sampled between 2003 and 2006 at various sites, along the Mediterranean coast (Marina smir, Nador) and on the Strait of Gibraltar (Ksar-sghir, Dalya, Belyounech) (Table 1). The taxonomic identification of species was done by experts in these fields, using standard literature and taxonomic keys. Voucher specimens of all species tested are deposited in the herbarium of our Laboratory of Applied Algology-Mycology, Department of Biology, Faculty of Sciences, Abdelmalek Essâadi University, 93002 Tetouan, Morocco (Table 1). Seven species belong to the Gelidiales order, nine to Gigartinales order, eight to Ceramiales order, one to Bonne-maisoniales order and one to Rhodymeniales.

Preparation of extracts

After collection, the samples were rinsed with sterile seawater to remove associated debris and necrotic parts. Epiphytes were removed from the algae and the surface microflora was removed by washing the algal samples for 10 min with 30% ethanol. The samples were shade dried, cut into small pieces and powdered in a mixer grinder. The powder obtained was preserved cold (-12°C). Samples (5 g) were extracted with methanol solvents for 8 h using a soxhlet apparatus. The resulting organic extracts were concentrated to dryness under reduced pressure at 30 - 35°C with a rotary evaporator. Each residue was weighed and stored in sealed vials in a freezer until being tested. All extracts were stored at (-4°C) (Ozdemir et al., 2004).

Bacterial strains

The strains used were, three gram-positive bacteria *Enterococcus faecalis* (ATCC 29212), *Enterococcus faecalis* (ATCC 29213) and *Staphylococcus aureus* (ATCC 25923) and two gram-negative bacteria *Escherichia coli* (ATCC 25922) and *Klebseila pneumoniae* (ATCC 700603). The bacteria strains were obtained from the Micro-biology Department, Faculty of Pharmacy, University of Grenade, Spain. All cultures were kept on Brain Heart Infusion (BHI, Sigma) agar plates and stored at 4°C, except the initial stock cultures which were stored at -80°C in BHI broth containing 20% glycerol.

Antibacterial activity by disc diffusion assay

The screening of the antibacterial activity of the methanolic extracts was performed by the disc diffusion technique in agar-plated petri dishes (Hellio et al., 2000). Sterile discs (BBLTM) of 6 mm were used. Methanol extracts (25 µl) were loaded on each of these discs, allowed to dry overnight at room temperature for 24 h to evaporate methanol, and then tested in antibacterial activity. A bacterial suspension (number 0.5 in McFarland scale about 1.5×10^8 bacteria ml^{-1}) was spread on Mueller-Hinton (pH 7.4) agar using a cotton swab. After incubation for 24 h at 37°C, the antibacterial activity was quantified as the diameter (mm) of the growth inhibition zones. Control paper discs with the solvent (100%) were tested for every assay and showed no antibacterial activity. All extracts were tested in three discs.

Statistical analysis

The data were statistically analysed by applying a one-way ANOVA for comparison of mean values. All tests were considered to be statistically significant at $P < 0.05$.

RESULTS

Out of 26 species of *Rhodophyceae* tested, 25 species (96%) showed an antimicrobial activity on at least on one of the micro-organisms. Concerning the distribution of this activity among the collected groups of seaweeds, our results show that activity one is present with all the orders of seaweeds (Table 2). Of the 25 active species, 12 of the species had an antibacterial activity against *E. coli* ATCC 25922, 8 of species against *K. pneumoniae* ATCC 700603, *E. faecalis* ATCC 29213, *E. faecalis* ATCC 29212 and 25 species inhibited the development of *S. aureus* ATCC 25923.

Crude extracts of *Hypnea musciformis, Halopitys incurvus* and *Gelidium pusillum* showed an inhibitory action against the five bacteria tested. The extracts of *H. incurvus* and *G. pusillum* showed the greatest inhibition on *S. aureus* and *E. coli*, moderate inhibition on *E. faecalis* and *K. pneumoniae*, whereas the extract of *H. musciformis* showed the highest inhibition on all the

Table 1. List of marine algae.

Order	Marine algae	Date of collection	Site of collection	Position
Ceramiales	*Alsidium corallinum*	15/05/2005	Nador	35º11'11.98''N 2º55'30.75''O
	Centroceras clavulatum	15/05/2005	Marina smir	35º45'56.81''N 5º20'58.04''O
	Callithamnion granullatum	03/10/2003	Ksar sghir	35º50'52.58''N 5º33'39.04''O
	Ceramium rubrum	10/08/2003	Ksar sghir	35º50'52.58''N 5º33'39.04''O
	Halopitys incurvus	15/06/2006	Dalya	35°54'24.20"N 5°28'18.84"O
	Osmundea pinnatifida	08/05/2006	Ksar sghir	35º50'52.58''N 5º33'39.04''O
	Pterosiphonia complanata	14/05/2006	Ksar sghir	35º50'52.58''N 5º33'39.04''O
	Boergeseniella thuyoides	12/08/2006	Ksar sghir	35º50'52.58''N 5º33'39.04''O
Gelidiales	*Gelidium attenatum*	14/05/2006	Dalya	35°54'24.20"N 5°28'18.84"O
	Gelidium latifolium	15/06/2006	Dalya	35°54'24.20"N 5°28'18.84"O
	Gelidium pusillum	11/01/2004	Marina smir	35º45'56.81''N 5º20'58.04''O
	Gelidium pulchellum	16/08/2005	Ksar sghir	35º50'52.58''N 5º33'39.04''O
	Gelidium sesquipedale	12/08/2006	Ksar sghir	35º50'52.58''N 5º33'39.04''O
	Gelidium spinulosum	12/05/2005	Ksar sghir	35º50'52.58''N 5º33'39.04''O
	Pterocladea capillacea	15/06/2006	Dalya	35°54'24.20"N 5°28'18.84"O
Gigartinales	*Chondrocanthus acicularis*	15/05/2005	Marina smir	35º45'56.81''N 5º20'58.04''O
	Caulacanthus ustulatus	15/05/2005	Marina smir	35º45'56.81''N 5º20'58.04''O
	Gracilaria confervoides	12/08/2006	Ksar sghir	35º50'52.58''N 5º33'39.04''O
	Gracilaria multipartita	12/08/2006	Ksar sghir	35º50'52.58''N 5º33'39.04''O
	Gymnogongrus patens	12/05/2005	Ksar sghir	35º50'52.58''N 5º33'39.04''O
	Hypnea musciformis	08/05/2006	Ksar sghir	35º50'52.58''N 5º33'39.04''O
	Plocamium cartilagineum	14/05/2006	Bel younech	35°54'34,87"N 5°23'41.91"O
	Plocamium coccineum	10/08/2003	Ksar sghir	35º50'52.58''N 5º33'39.04''O
	Sphaerococcus coronopifolius	10/04/06	Bel younech	35°54'34,87"N 5°23'41.91"O
Bonnemaisoniales	*Asparagopsis armata*	15/06/2006	Dalya	35°54'24.20"N 5°28'18.84"O
Rhodymeniales	*Rhodymenea pseudopalmata*	16/08/2005	Ksar sghir	35º50'52.58''N 5º33'39.04''O

Table 2. Antimicrobial activities of the seaweeds species by disc-diffusion technique.

Order	Methanolic extracts	*E. coli* ATCC 25922	*K.pneumoniae* ATCC 700603	*E. faecalis* ATCC 29212	*E. faecalis* ATCC 29213	*S. aureus* ATCC 25923
Ceramiales	*A. corallinum*	11.67±0.57	10.00±0.00	-	-	16.67±0.57
	C. clavulatum	-	-	-	-	15.00±0.00
	C. granullatum	-	-	-	-	19.00±0.00
	C. rubrum	16.00±0.00	-	12.33±0.57	-	25.33±0.57
	H. incurvus	15.33±0.57	11.67±0.57	11.67±0.57	10.00±0.00	24.67±1.15
	O. pinnatifida	-	-	-	-	-
	P. complanata	9.00±0.00	-	14.33±0.57	16.00±0.00	20.67±0.57
	P. thyoides	-	-	-	-	13.00±0.00
Gelidiales	*G. attenatum*	11.67±1.52	10.67±0.57	-	10.00±0.00	19.00±0.00
	G. latifolium	-	-	-	-	14.67±0.57
	G. pusillum	16.33±0.57	9.00±1.00	12.67±0.57	9.00±0.00	26.67±0.57
	G. pulchellum	8.67±0.57	-	15.00±1.00	-	20.00±0.00
	G. sesquipedale	7.67±0.57	-	-	-	18.0±0.00
	G. spinullosum	19.67±0.57	-	15.33±0.57	-	32.67±0.57
	P. capillacea	-	-	-	-	13.00±0.00
Gigartinales	*C. acicularis*	10.33±2.08	15.67±1.15	-	8.33±0.57	16.33±0.57
	C. ustulatus	-	9.00±0.00	-	-	14.33±0.57
	G. confervoides	-	-	-	-	13.00±0.00
	G. multipartita	-	10.00±0.00	-	-	17.67±0.57
	G. patens	-	-	-	-	10.00±0.00
	H. musciformis	20.67±0.57	19.00±0.00	24.33±1.15	15.33±0.57	35.00±1.73
	P. cartilagineum	8.00±0.00	-	11.67±0.57	13.00±0.00	23.33±0.57
	P. coccineum	-	-	-	-	15.67±0.57
	S. coronopifolius	-	-	-	9.00±0.00	13.33±0.57
Bonnemaisoniales	*A. armata*	-	-	-	-	20.33±0.57
Rhodymeniales	*R. pseudopalmata*	-	-	-	-	12.67±0.57

The activity is classified according to the diameter of the zone of inhibition (in mm ±SD) around the sample's point of application. Weak inhibition: ≤10 mm, 10 mm < moderate inhibition ≤ 15 mm, highest inhibition: > 15 mm, - : no activity.

microorganisms tested, with inhibition diameters ranging from 15 to 35 mm.

All extracts of algae tested except *Osmundea pinnatifida* were effective against the gram-positive strain S. *aureus*, which was highly inhibited as by the extracts *H. musicformis*, *Gelidium spinulosum, G. pusillum and Pterosiphonia complanta*; the diameter of the inhibition zone was 35, 32, 26 and 20 mm, respectively (Figure 1). Rizvi and Shameel (2005) reported that the methanolic extract of the alga *O. pinnatifida* did not inhibit the *S. aureus* and *K. pneumoniae* bacteria. This result is in agreement with the present study. The diameter of the zone of inhibition varies from one algal extract to the other and for the same extract, from one microorganism to the other (Figure 2).

In our findings, all the extracts were active on S. *aureus* ATCC 25923 with the extract of *H. musciformis* showing the greatest inhibition on all the bacteria tested except on the bacteria *E. faecalis ATTC* 29213, on which it showed a moderate inhibition (Table 2). The inhibition effects from extracts are classified as greatest at weak inhibition as follows, for *E. coli*: *H. musciformis, G. spinullosum, G. pusillum, Ceramium rubrum,* and *H. incurvus* > *Alsidium corallinum, Gelidium attenatum,* and *Chondracanthus acicularis* > *Pterosiphonia complanata, Gelidium pulchellum,* and *Plocamium cartilagineum.* For *K. pneumonia*: *H. musciformis,* and *C. acicularis* > *H. incurvus,* and *G. attenatum* > *A. corallinum, Gracilaria multipartita, Caulacanthus ustulatus,* and *G. pusillum.* For *E. faecalis* ATCC 29212: *H. musciformis,* and *G. spinullosum* > *G. pulchellum, P. complanata, G. pusillum, C. rubrum, P. cartilagineum,* and *H. incurvus.* For *E. faecalis* ATCC 29213: *P. complanata,* and *Hl musciformis* > *Pl cartilagineum, H. incurvus,* and *G. attenatum* > *G. pusillum, Sphaerococcus coronopifolius,* and *C. acicularis.* For *S. aureus*: *H. musciformis, G. spinullosum, G. pusillum, C. rubrum, H. incurvus, P. cartilagineum, P. complanata, Asparagopsis armata, G. pulchellum, G. attenatum, C. granullatum, G. sesquipedale, G. multipartita, A. corallinum, C. acicularis,*

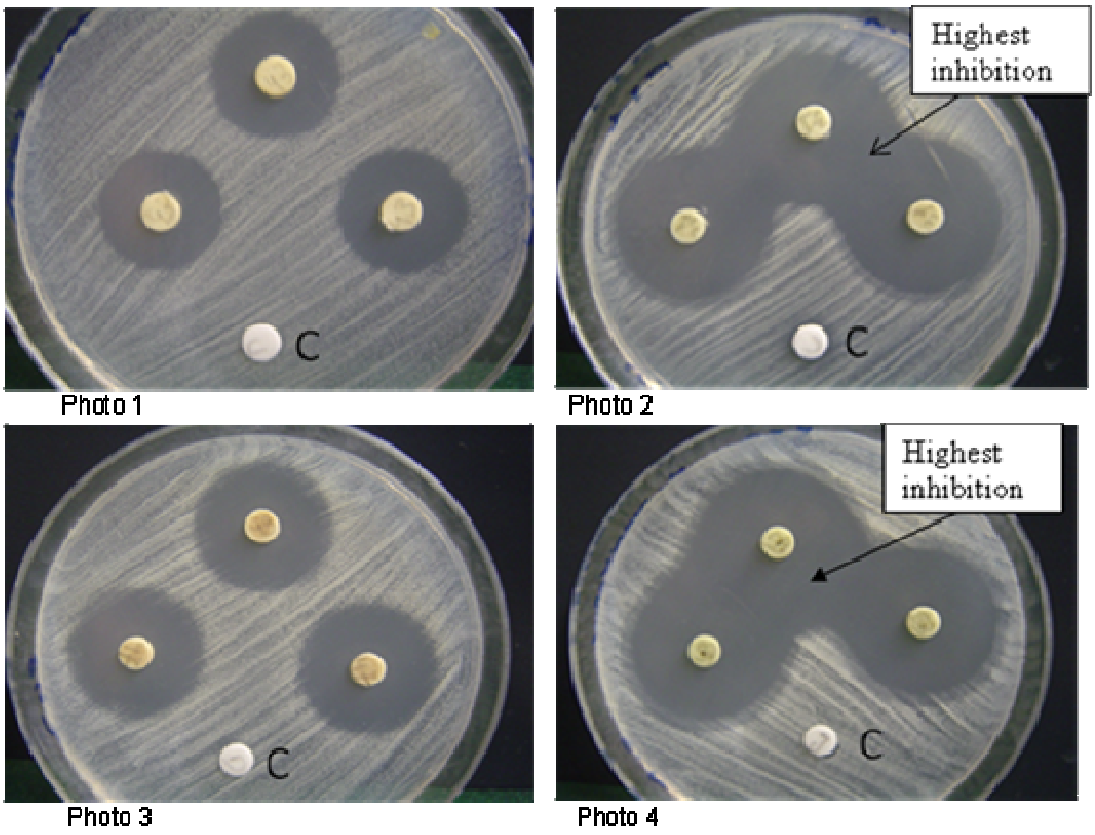

Figure 1. The effect of the methanolic extracts of *P. complanata* (Photo 1), *G. spinullosum* (Photo 2), *G pusillum* (Photo 3) and *H. musciformis* (Photo 4) on *S. aureus*. C: negative control.

and *Plocamium coccineum* > *Centroceras clavulatum, Gelidium latifolium, Caulacanthus ustulatus, S. coronopifolius, Boergeseniella thuyoides, Pterocladea capillacea, Rhodymenia pseudopalmata,* and *Gymnogongrus patens*.

According to these results, it appears that the bacterium *S. aureus* is more sensitive to the methanolic extracts compared to the other bacteria tested, whose great zones of inhibition were shown by the extract of *H. musciformis* and *G. spinullosum*.

DISCUSSION

The antibacterial activity was screened against widely-distributed *E. coli*, *E. faecalis*, *S. aureus* and *K. pneumoniae* pathogens, which cause serious problems for human health. The bacterial strain *S. aureus* is responsible for food poisoning, suppurating located infections and blood-poisoning for debilitated subjects. This study has demonstrated that algae extracted by a methanol solvent showed the greatest inhibition diameters. These results are in agreement with the observations of Vlachos et al. (1996), Gonzalez et al. (2001), Ozdemir et al. (2004), Karabay-Yavasoglu et al. (2007), Taskin et al. (2007) and, Kandhasamy and Arunachalam (2008), who reported that extracts prepared with methanol showed the best activity. Taskin et al. (2007) reported that the highest activity was shown on *E. coli* ATCC (29998) (32.00 ± 1.73 mm) and *E. faecalis* ATCC (8043) (21.66 ± 0.57 mm) by a methanolic extract of *Corallina officinalis* red algae. Kandhasamy and Arunachalam (2008) observed that methanolic extract of *H. musciformis* is active on the *K. pneumoniae* (13 ± 0.59), *S. aureus* (12 ± 0.69) and *E. faecalis* (12 ± 0.75) bacteria. These results, however, are in contrast with those of Bansemir et al. (2006), who mentioned dichloromethane as the most suitable solvent and those of Lima-Filho et al. (2002), who reported that hexane was the best solvent for extracting antibacterial substances from algae.

Magallanes et al. (2003) reported that the bacterium *E. faecalis* ATCC (29212) was inhibited by the ethanolic extract of *Grateloupia doryphora* (15.7 mm), whereas the bacterium *E. coli* ATCC 25922 was inhibited by ethanolic extracts of *Rhodophyceae* from *Grateloupia doryphora*, *Rhodymenia flabellifolia*, *Gracilariopsis lemaneiformis*, *Ahnfeltiopsis durveillaei*, *Porphyra columbina* and *Cryptopleura sp*. While Sastry and Rao (1994) showed that the methanolic extract of *Gracilaria corticata* red algae was negative on the bacteria *E. coli* ATCC 25922, a chloroform

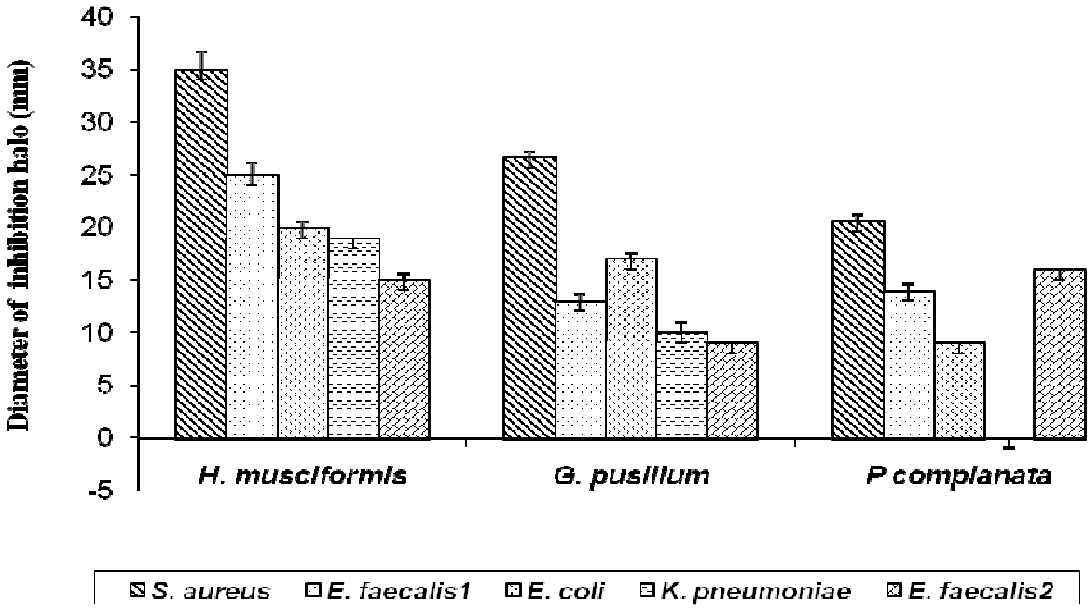

Figure 2. Diameter averages (mm) of the zones of inhibition of methanolic extracts of *H. musciformis, G. pusillum* and *P. complanata* against five tested bacterial strains. *E. faecalis* 1: *E. faecalis* ATCC 29212 and *E. faecalis* 2: *E. faecalis* ATCC 29213.

extract had moderate bactericidal activity and a benzene extract had a weak inhibition potential. The three extracts: methanolic, chloroform and benzene of *Acanthophora delilei* did not exhibit any inhibition on this bacterium.

Several studies have detected the antibacterial activities from red algae, the study of Gonzalez et al. (2001) reported that the methanolic extracts of *H. incurvus* and *P. capillacea* did not cause any inhibition of the bacterium *S. aureus* MB5393. Etahiri et al. (2003) mentioned that the methanolic extracts of *H. incurvus*, *H. musciformis*, *P. cartilagineum*, *S. coronopifolius* and *G. latifolium* showed an inhibition on the bacterium *S. aureus* (ATCC 6538) and the result was negative for the extract of *A. armata* on this bactaria. The methanolic extracts from *H. incurvus*, *S. coronopifolius*, and *A. armata* did not show any activity on the bacterium *E. coli* (ATCC 10536). The methanolic extract of the seaweed *H. musciformis* exhibited strong antibacterial activity against the gram positive and seven gram negative bacteria (Siddqiui et al., 1993). Bansemir et al. (2006) investigated the antibacterial activities of extracts from 26 algae species prepared with dichloromethane against five fish-pathogenic bacteria. He reported that the most active algal species was *A. armata* against all tested bacteria. The dichloromethanolic extracts of *H. incurvus* and *C. rubrum* showed their highest activity against the marine bacterium *Pseudomonas anguilliseptica*, while the extract of *P. cartilagineum* showed a weak activity on these five fish-pathogenic bacteria. The extract methanol-dichloromethane (1:1) of *H. musciformis* inhibited the growth of gram positive strains (*Bacillus cereus*, *Bacillus subtilis* and *Micrococcus luteus*,) to the extent of 66.0% at 30°C, whereas all the gram positive bacteria were susceptible at 20°C (Selvin and Lipton, 2004). The antibacterial activity of methanol/toluene (3:1) extract from *G. pusillum* and *S. coronopifolius* did not show any inhibition on strains of *E. coli* and *B. subtilis* (Ballesteros et al., 1992). On the other hand, the observations of Caccamese et al. (1980, 1981) reported that, methanol-toluene (3:1) extracts from *S. coronopifolius* and *P. cartilagineum* inhibit the bacteria strain *B. subtilis*.

The higher frequency of activity against gram-positive bacteria has also been observed in most of the surveys on antimicrobial activities from seaweeds reported in literature (Gonzalez et al., 2001; Etahiri et al., 2003). The present investigation revealed that *S. aureus* was more sensitive than all the extracts, with the largest inhibition diameter. It was reported that the gram-positive bacterial strains were more susceptible to seaweed extract than gram-negative bacterial strains (Ballantine et al., 1987). Similar results were obtained by Ballantine et al. (1987), Rossell and Srivastava (1987) and Magallanes et al. (2003).

In the literature, the majority of the compounds responsible for the antibacterial activity at the marine algae are of terpenic (König et al., 1999a; Etahiri et al., 2001; Wang et al., 2007; Smyrniotopoulos et al., 2008; Vairappan et al., 2008; Chakraborty et al., 2010), phenolic (Xu et al., 2003; Oh et al., 2008; Etahiri et al., 2007) and lipidic nature (Findlay and Patil, 1986; Sidqqiui et al., 1993; Al-Fadhli et al., 2006; Paul et al., 2006). Two bromoditerpenes were isolated from *S. coronopifolius*, 12 S-hydroxybromospha-erodiol and bromosphaerone; these compounds showed an antibacterial activity against the

bacteria gram positive like *S. aureus* with an inhibiting minimal concentration of 0.104 and 0.146 µM, respectively (Etahiri et al., 2001). The phenolic compound 3,4,6-tribromo-5-methoxymethyl-benzene-1, 2-diol which was found at the species *P. complanata* proved to have an antibacterial activity against several positive and negative pathogenic bacteria gram. Its inhibiting minimal concentration against the bacterium *S. aureus* was 2.8 µg/ml (Etahiri et al., 2007), and thus, the fatty-acids which were found in the extract methanolic of the alga *H. musciformis* exhibited strong antibacterial activity against the gram positive and seven gram negative bacteria (Sidqqiui et al., 1993).

Differences between the results of the present investigation and those of other studies may be due to the organic solvents used for the extraction of bioactive compounds and the differences in the assay methods, the geographical zone and the seasonal production of bioactive compounds. Salvador et al. (2007) studied the antimicrobial activities of 82 marine algae in fresh and lyophilized forms and according to a seasonal variation, they reported that red algae had both the highest values and the broadest spectrum of bioactivity. The highest percentages of active extracts of *Phaeophyceae* and *Rhodophyceae* were found in the autumn, whereas, they were found in the summer for *Chlorophyceae*. Etahiri et al. (2003) showed that algae extracts collected in the spring were significantly more active than those collected in the winter.

According to these reports, and taking into account the results detailed in the present contribution, it appears that the seaweeds from our coasts possess significant bioactive capacities, and thus deserve a place in marine biotechnology programmes to examine the properties of natural products. The extracts of *H. musciformis*, *G. spinullosum*, *C. rubrum*, *H. incurvus*, *P. complanta* and *G. pusillum* showed a real potential with good yields. These results suggest the possibility of using marine algae extracts in therapy as natural alternatives to antibiotics currently in the market, and clearly show that seaweeds from the Mediterranean coast of Morocco are valuable source of biologically active compounds. Further research is underway to determine the structure and nature of these antibacterial substances.

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Full Length Research Paper

Effect of alternating and direct currents on *Pseudomonas aeruginosa* growth *in vitro*

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Eradication of pathogenic bacteria from important part of our life such as dental tools, foods and wounds is necessary. Based on the effect of natural selection, these bacteria become resistant to antibiotics. In some cases such as the section where burnt are treated in the hospital, we observe high rate of mortality as well as high numbers of resistant bacteria. In order to solve these problems, electrical stimulation (ES) is proposed. This has being shown to be an effective method. One of the reasons why it works could be due to the bacteria static property of electrical stimulation. So, more studies must be done so as to reach optimum voltage and currents. The test media were Muller-Hinton agar and eosin methylene blue (EMB) agar. In this research *Pseudomonas aeruginosa* which was isolated from patients' wounds was examined with levels of alternating and direct current (AC and DC) electrical stimulation (1.5V, 3.5V, 5.5V and 10V) to see if these currents could inhibit *P. aeruginosa* growth *in vitro*. The experiment was performed in two forms: The first was carried out immediately while the second was carried out 19 h after being cultured. Different patterns of zone of inhibition were observed in the two forms of our research. AC current had low inhibitive effect on *P. aeruginosa*'s growth. Anode and cathode showed different zone of inhibition, in each of the forms and media. The maximum inhibition zone (22 mm) was observed around cathode in 3.5 V direct current which was immediately used in the media. Direct current significantly inhibits growth of *P. aeruginosa*. Based on other studies on different bacterial species, ES can be applied to sterilization and controlling of superficial infections like in burnt patients.

Key words: Electrical stimulation, *Pseudomonas aeruginosa*, wound healing.

INTRODUCTION

Electrical stimulation (ES) has been used for hundreds of years for a variety of purposes including muscle strength training and wound healing. Promoting wound healing is possible with some mechanisms including increased circulation (Petrofsky et al., 2005; Kloth, 2002), increased angiogenesis (Bai et al., 2004; Zhao et al., 2004; Ojingwa and Isseroff, 2003) increased proliferation of epidermal tissue and antibacterial effect of ES (Rowley et al., 1974). Bacteria static effect of electrical stimulation was for the first time reported over 30 years ago by Rowley et al. (1974). Inhibition of bacterial growth has been reported by other researchers who used different types of electrical stimulation (Merriman et al., 2004; Kincaid and Lavoie, 1989; Petrofsky et al., 2008). Two main types of electrical stimulation were used in these studies: High-voltage pulsed current which is extremely painful for the patients and in which the duration of treatment with this ES is short and the second, low intensity direct current with opposite properties. Based on the effect of natural selection, bacteria become resistant to antibiotics. In some cases such as the section where burnt are treated in the hospital, we observe high rate of mortality as well

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Abbreviations: AC, Alternative current; **DC,** direct current, **EMB,** eosin methylene blue; **ES,** electrical stimulation.

Figure 1. Experimental setup, glass petri dish with stainless-steel electrodes connected to DC power supply before placement into incubator

as high number of resistant bacteria. So, we can use ES as a common antibacterial against these resistant bacteria. We can also use ES as a common sterilizer to sterile dental tools, food, etc.

ES with different waveforms, voltages, etc have different effects on bacteria growth. So, it provides a vast field for research. Some authors showed wound healing with DC micro currents while others showed only healing with strong AC currents (Feedar et al., 1992; Franek et al., 1999; Houghton et al., 2003). Report has being made to show that in the early stages of wound healing, the polarity needs to be positive around the wound and negative away from the wound, but there are other reports contrary to this (that is, the polarity needs to be negative around the wound and positive away from the wound) (Yarkony, 1994; Bogie et al., 2000; Demir et al., 2004). Due to differences in results and types of ES, more research needs to be done to reach an optimum and standard methods. In addition, for various purposes, we need to find different standard methods.

In this study, we used different voltages and media culture. We did comparable studies of both AC and DC effect on bacteria growth. We also gave ES once immediately after culture and in the other form, 19 h after culture to investigate their different effects on bacterial growth.

MATERIALS AND METHODS

Organisms tested

Pseudomonas aeruginosa (gram-negative rod) which were used in this study were isolated from patients in Motahari hospital laboratory, Iran.

Procedures and instrumentation

Sterile glass Petri dishes were used throughout the experiment. Two holes were bored through each glass 100 mm Petri dishes (7 mm diameter and 45 mm apart). Stainless-steel wires were used as electrodes (0.44 mm gauge and 23.20 mm length). Sterile electrodes were covered by plastic to prevent the wires from rolling and breaking contact with the medium. All tools were sterilized by autoclaving before using. We used DAZHENG DC power supply (PS-303D) and ALFA step-down transformer as the ES devices.

We studied antibacterial effect by Kirby-Bauer technique (Bauer et al., 1996) which was standardized by the National Committee for Clinical Laboratory Standards (NCCLS) instruction with one difference; instead of using standard antibiotic discs, we used ES. The test media selected were Muller-Hinton agar and eosin methylene blue (EMB) agar. Muller-Hinton agar was used in the semi quantitative Kirby-Bauer technique for determining effectiveness of antibiotics due to the consistency of the widths of zones indicating inhibition of bacterial growth (Kincaid and Lavoie, 1989). To run the test, wires in the Petri dishes were connected by alligator-clip leading to ES devices. The experimental setup is shown in Figure 1. Voltages of 1.5, 3.5, 5.5 and 10 were applied to the test organisms. In the first type of our study, each plate was incubated at 37°C for 19 h following exposure to ES. In the second type, each plate was incubated at 37°C for 19 h and then was exposed to ES for 19 h at 37°C. The width of the zone of inhibition parallel with the wire electrodes where no bacterial growth occurred was measured with a millimeter ruler. Test results represent the average of two measurements per zone of inhibition in each plate. Changes in the pH of the media were determined by using pH paper touched to the surface of the media. By measuring the voltage and the resistance of the medium, we found out that by increasing the voltage, the resistance of the medium decreases, so the conductivity of the medium would increase. Two kinds of currents were used in this study. The first current which was used in the first stage is the DC signal. The frequencies of these signals are zero and the amplitude of DC

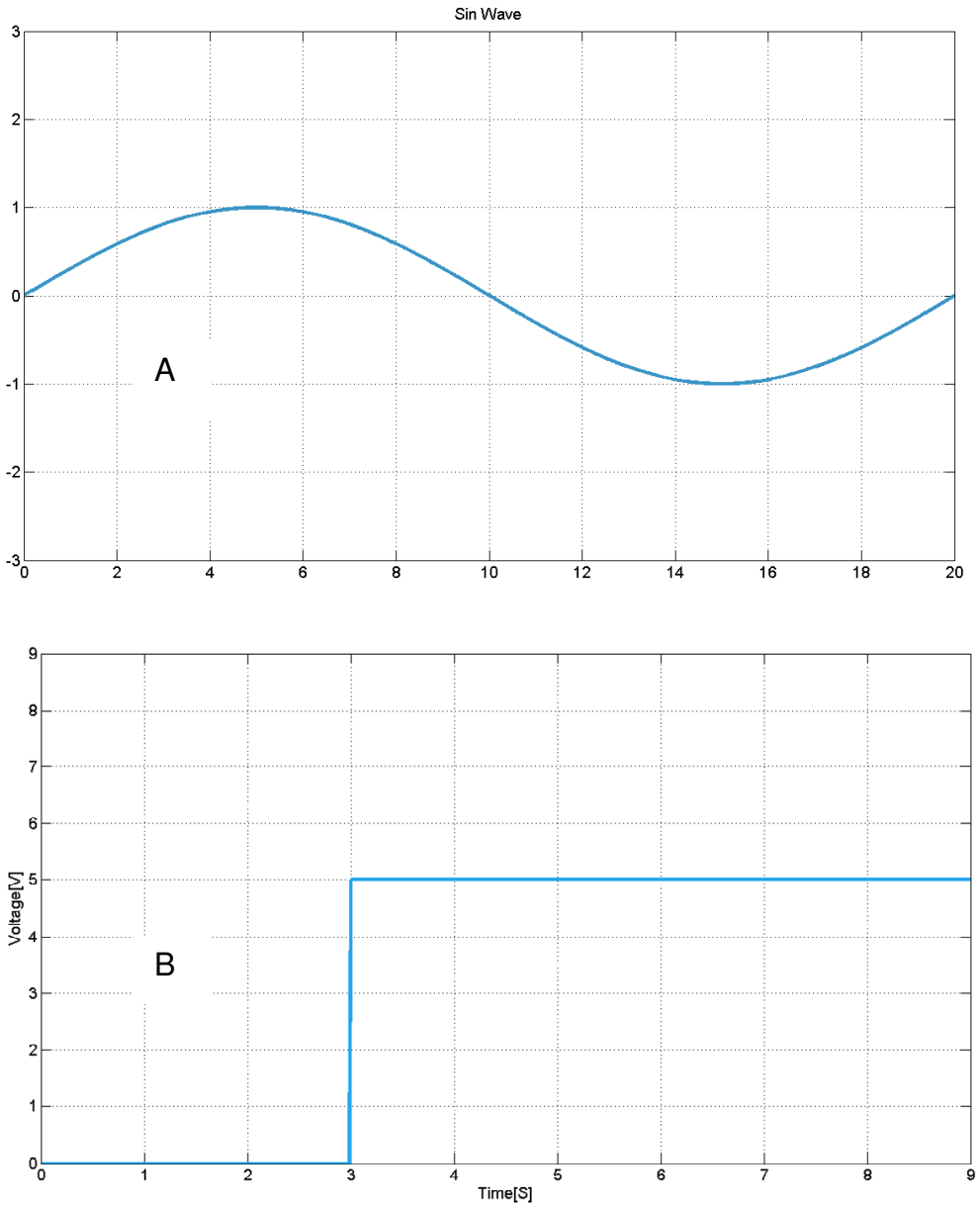

Figure 2. Comparison of waveforms. (a) AC signals with 50 HZ frequency and 20 ms period (b) DC signals with zero frequency.

signals is variable. The second current used in the second stage is AC signals. There are many types of AC signals, but in this study sine waves was used as AC signals with 50 HZ frequency and 20 ms period (Figure 2).

Controls

Current was sent through sterile media without organisms and then bacteria were cultured on media to determine the presence of potential toxic electrochemical products from the interaction of current, wire and media. Seeded plates with the wire electrodes in place were incubated without exposure to ES to determine whether the wires themselves, the media, or a combination of both would be inhibitory to bacterial growth. Seeded plates with the wire electrodes in place were incubated without exposure to ES and organisms to ensure sterility.

Data analysis

Results were analyzed by two-way analysis of variance (ANOVA). Regression analysis was preformed by using the Microsoft Excel 2007 and MATLAB 2009B.

RESULTS

We have four variables: (1) Type of ES (AC and DC)

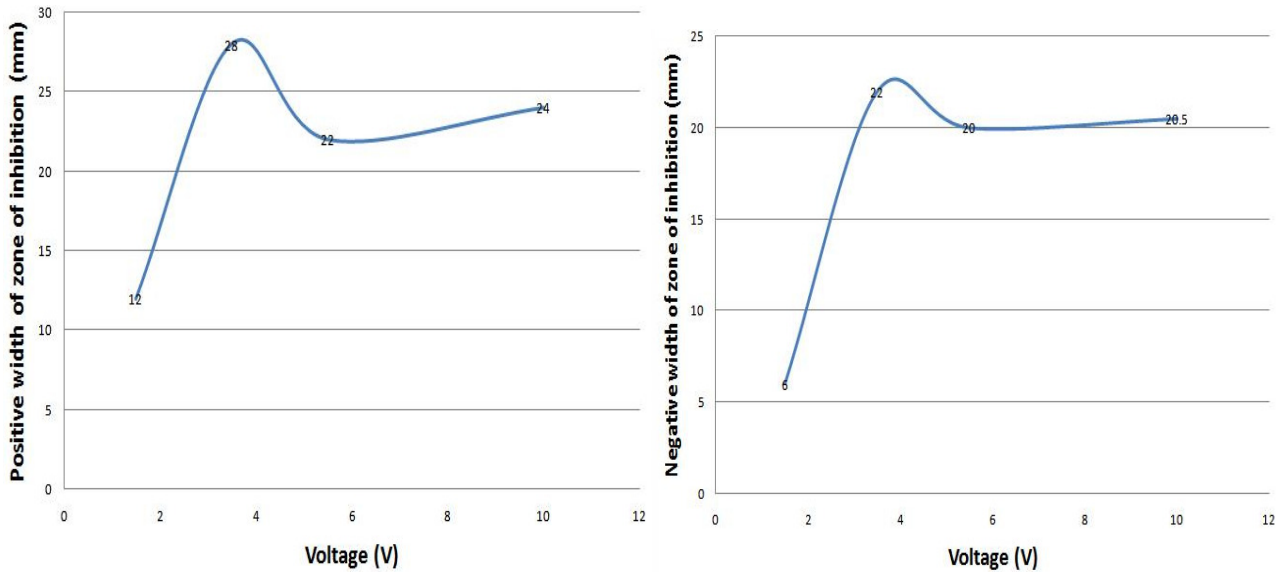

Figure 3. Inhibitory zone of Mueller-Hinton agar with DC (type 1).

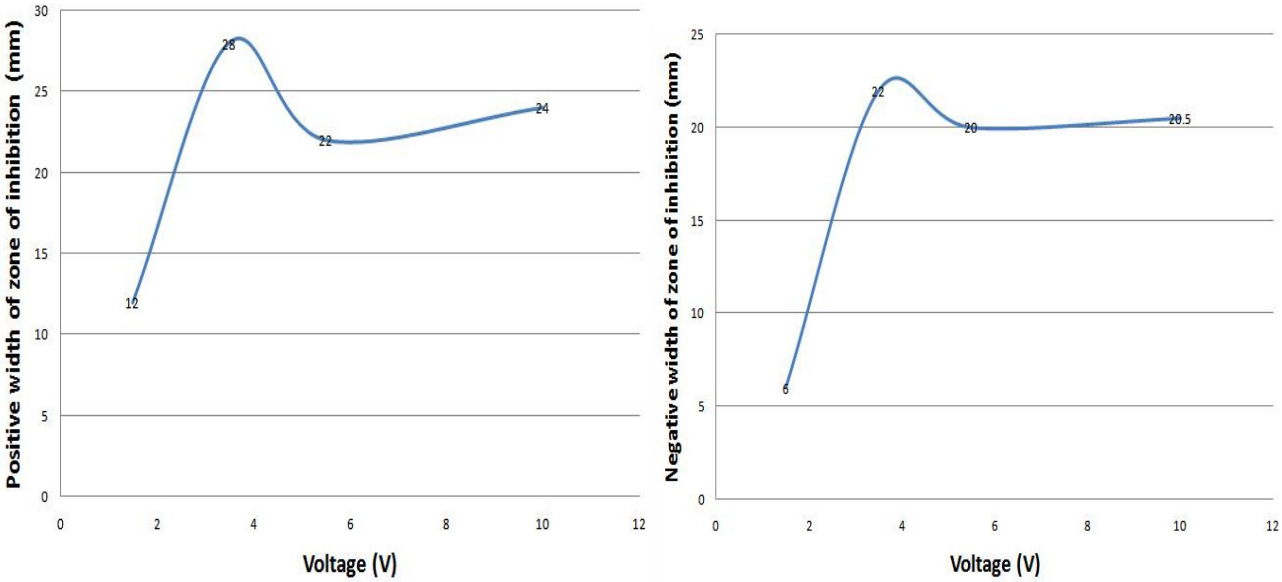

Figure 4. Inhibitory zone of Mueller-Hinton agar with DC (type 2).

treatment; (2) electrode polarity; (3) type of media culture; (4) different voltages. ANOVA results revealed that there are significant statistical differences with the type of ES (DC and AC), ($p < 0.05$), media culture and different voltages ($p < 0.05$ and $p < 0.01$). Statistical analysis revealed no significant differences with electrode polarity. Several control plates established that exposure to the current was the cause of the bactericidal effect on the test organisms. Sending current through sterile media without organisms and then culturing bacteria on media showed no inhibition of growth around the cathode, but the test organisms would not grow around the anode because of the presence of toxic electrochemical products. Plates containing seeded medium that were not exposed to ES showed no inhibition of growth, indicating that the wires themselves, the medium, or both, had no inhibitory effect. Seeded plates with the wires electrodes in place without exposure to ES and organisms displayed that the sterling method was prefect and no bacteria growth was observed. Growth inhibition of *P. aeruginosa*, represented by the measurements of the zones of inhibition, is shown in Figures 3, 4, 5, 6 and 7. In other cases with AC stimulation, we did not see any inhibitory effect on bacterial growth.

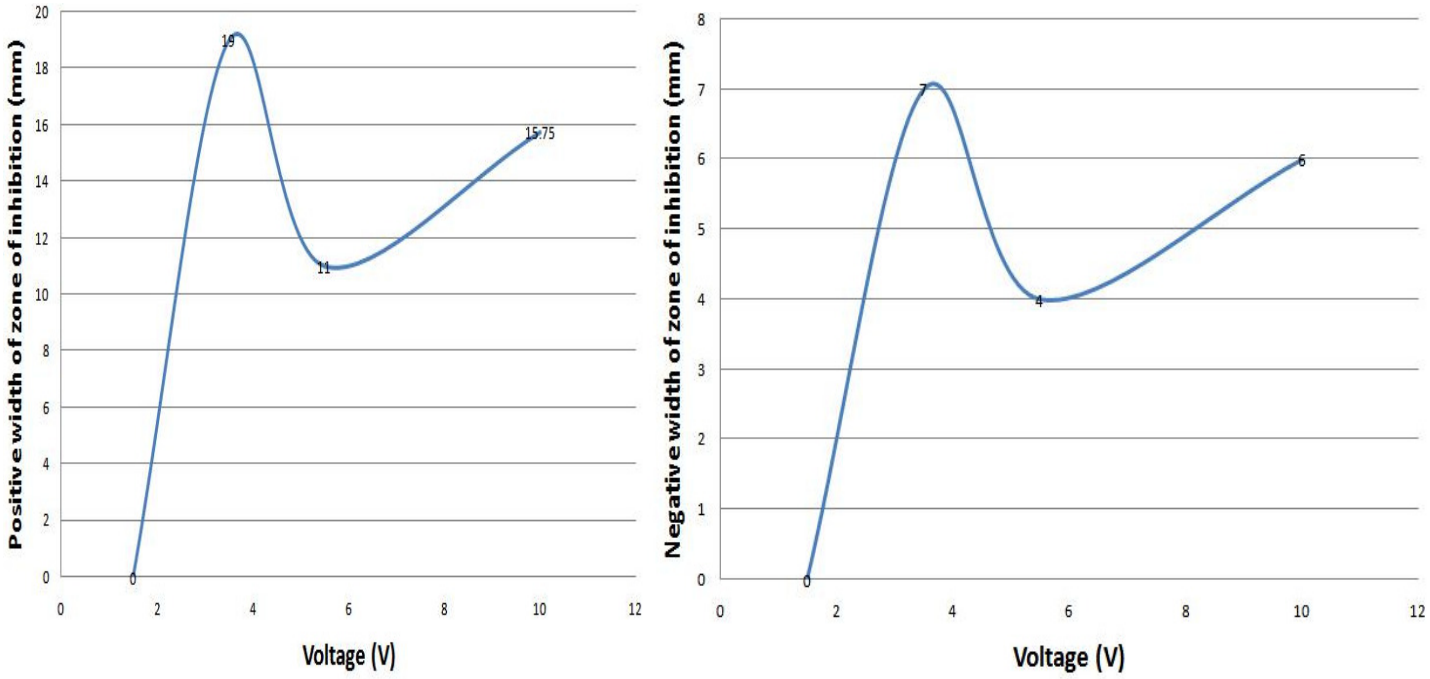

Figure 5. Inhibitory zone of EMB agar with DC (type 1).

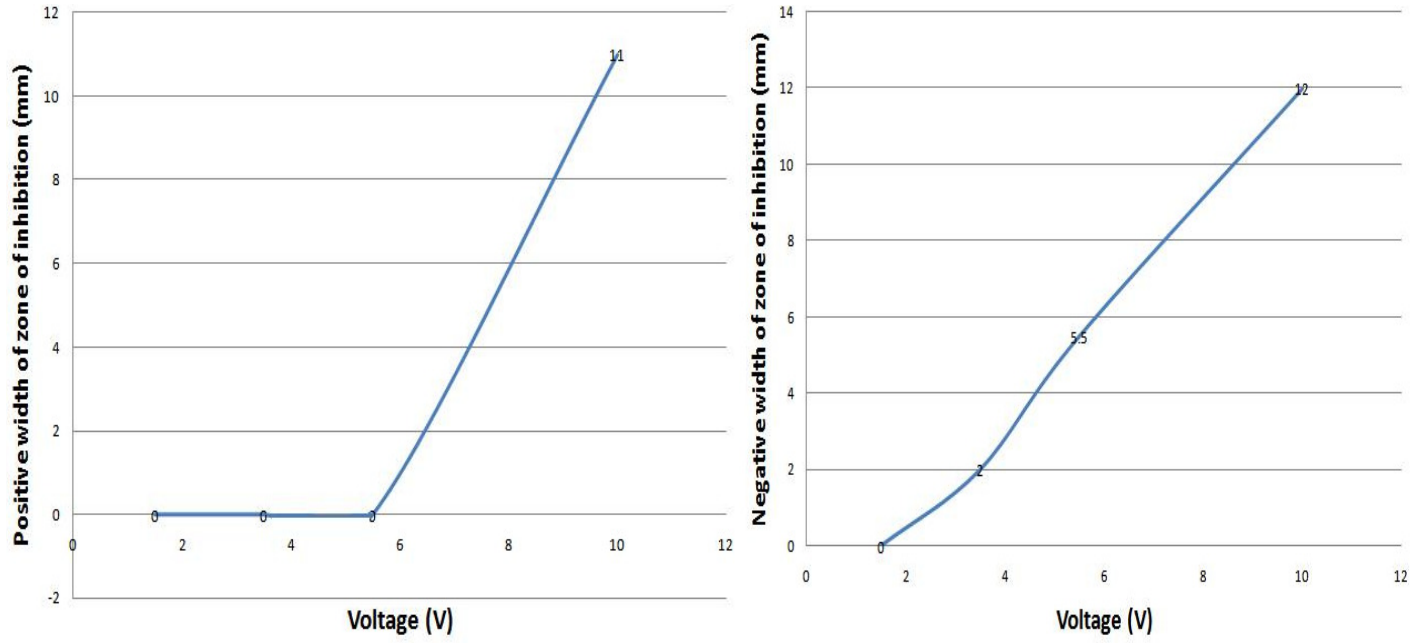

Figure 6. Inhibitory zone of EMB agar with DC (type 2).

DISCUSSION

Direct current can be effective in killing common wound infecting bacteria *in vitro*. One of the advantages of this study compared with other studies is that we did comparable studies of both AC and DC effect on bacteria growth. The data analysis revealed that there are statistically significant differences between the effect of DC and AC on bacteria growth. According to the averages obtained, DC has better antibacterial effect than AC.

Data analysis revealed that there are no significant statistical differences between cathode and anode. Effects

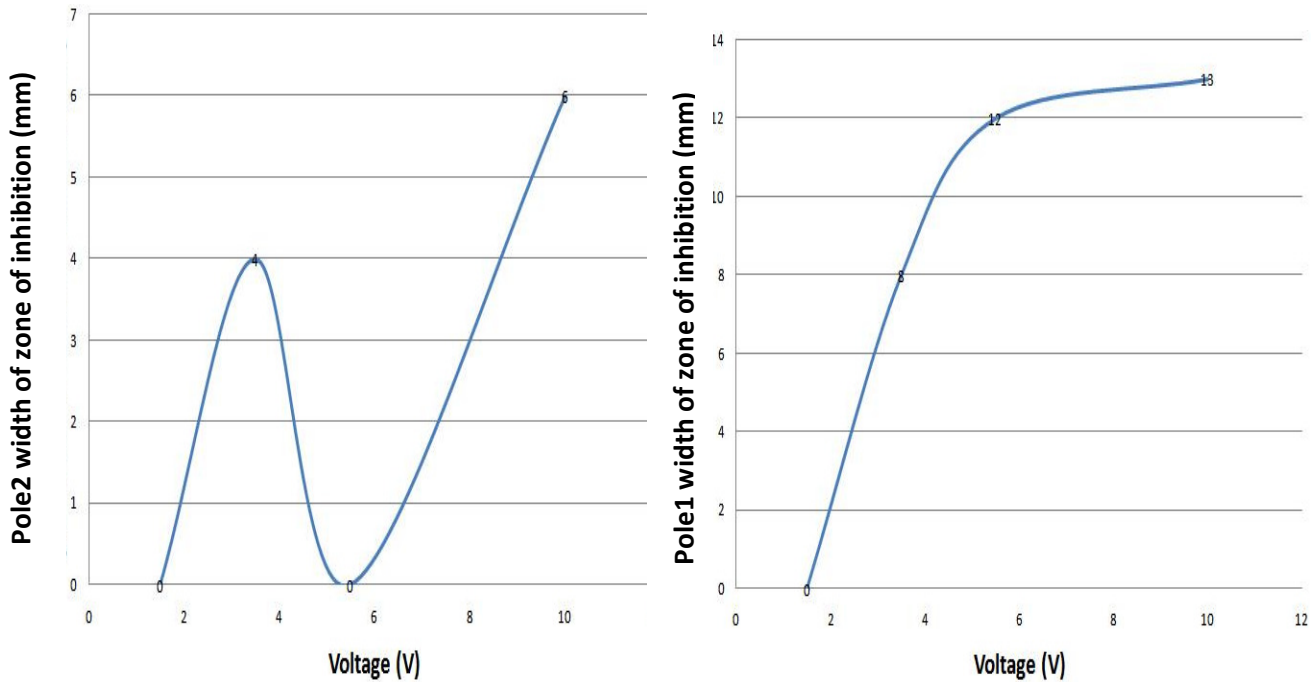

Figure 7. Inhibitory zone of Mueller-Hinton agar with AC (type 1).

of ES around the anode were complicated by the production of some toxic electrochemical end products created by passing current through the wire. These results are similar to those reported by Kincaid and Lavoie, (1989). But, we must notice that at 1.5 and 3.5 voltages in EMB agar we did not observe any electrochemical toxic products around the anode. Growing bacteria at the cathode end after sending current through sterile media without organisms suggest that no permanent change had occurred there. No bacteria growth at the anode end was observed after sending current through sterile media without organisms suggesting that lethal end products had accumulated and persisted. On the other hand, anode is painful to the patient if use for a long time unlike cathode. The other advantages of this study in comparison with other studies is that in this study we use two different media culture to see if there is differences in results by changing the media culture. The data analysis revealed that there are significant statistical differences between two media culture. So, we observed that with changes in the media, results would change because of different properties of media culture. So, this effect must be shown in *vivo* or in clinical wounds. In this study we used different voltages to reach an optimum voltage. The data analysis revealed that there are significant statistical differences between voltages. According to the averages obtained in method one of our study (that is, immediate exposure to ES), 3.5 V DC is the best voltages for bacteria growth inhibition (in both media cultures and around both electrodes). In method two of our study (that is, exposure to ES 19 h after culture) the averages obtai-ned, 5.5 V DC is the best in Mueller-Hinton agar (around the both electrodes) and 10 V DC is the best in EMB agar (around the both electrodes). If we consider method one as prevention and method two as treatment, we can use ES for both purposes.

Exposure to ES at the cathode has sufficient inhibitory effect on bacteria growth. Since cathode does not induce pain to the patient, it can be use for a long time. In this study, we suggest that treatment duration of 19 h or less than 19 h a day with 3.5 V DC and negative polarity around the wound and positive polarity away from the wound have better inhibitory effect on bacteria growth than other forms.

Alternatively, the actual current flow through the Petri plates was very low because of resistance from the test medium. If there is less resistance to current flow in human skin, then lower settings might have bactericidal effect in a clinical setting (Merriman et al., 2004). Futher investigations can be done *in vivo* on the application of DC with different voltages at both polarity and AC with different waveforms when their inhibitory effect on bacteria growth had been proved *in vitro*. Other investigation can include the effect of other type of electrical current on microorganisms' growth *in vitro*.

Conclusion

The purpose of this investigation is to study the effect of different AC and DC voltages with different conditions on bacterial growth. A statistically significant bacterial inhibi-

tory effect was found for direct current with different voltages. Because of, low intensity direct current inhibitory effect on bacterial growth according to these findings, we can practically use low intensity direct current to promote wound healing. These findings must be shown *in vivo* or in clinical wounds.

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Full Length Research Paper

Production of Extracellular Aspartic Protease in Submerged Fermentation with *Mucor mucedo* DSM 809

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Fungal milk-clotting enzymes have gained value as bovine Chymosin substitutes in the cheese industry. In this work, the effects of culture conditions on the production of extracellular milk clotting enzymes from *Mucor mucedo* DSM 809 in submerged fermentation were studied. The maximum activity was observed after 48 h of cultivation at 24°C in Erlenmeyer flasks. The optimized initial pH and shaking speed for enzyme production were 4.5 and 220 rpm, respectively. Glucose at a concentration of 1% (w/v) was the best carbon source for the production of enzyme among the carbohydrates examined (glucose, fructose, lactose, maltodextrin). On the other hand casein at a concentration of 0.5% (w/v) was the selected nitrogen source in the media formulation. Under optimized conditions enzyme levels reached 130 SU per ml fermentation broth. The inoculum type and size has also affected biomass production and the biosynthesis of the enzyme. The preferred method was the inoculation of the culture media with spores at a total load of $6x10^5$ spores per flask.

Key words: Milk clotting enzyme, Aspartic protease, *Mucor mucedo*, Sub-merged fermentation.

INTRODUCTION

Aspartic proteases are a group of proteolytic enzymes produced by many microorganisms. A significant property of aspartic proteases is the ability to coagulate milk, as is evidenced by their widespread application in the dairy industry to coagulate casein during the manufacturing of cheese. Calf rennet has been traditionally utilized for milk coagulation and curd formation. Milk clotting by calf rennet occurs essentially by cleaving the Phe_{105}-Met_{106} bond of *k*-casein, resulting in the release of a hydrophilic (macro) glycopeptide, which passes into the whey. Para-*k*-casein becomes positively charged at neutral pH and causes decrease of the repulsive forces between casein micelles thereby causing aggregation and curd formation (Vishwanatha et al., 2009).

The limited supply of natural calf rennet has simulated the search for alternative sources of milk-clotting enzymes of microbial origin and the production of recombinant bovine chymosin. Studies were mainly focused on fungal aspartic proteases although a few reports on bacterial enzymes can be found (Dutt et al., 2008). Filamentous fungi are the major producers of microbial aspartic proteases. Fungal substitutes for animal proteases proposed include those from *Rhizomucor pusillus* (Ismail et al., 1984), *Rhizomucor miehei* (Preetha and Boopathy, 1994; Escobar and Barnett, 1993), *Mucor bacilliformis* (Areces et al., 1992; Venera et al., 1997; Fernández-Lahore et al., 1997; Machalinski et al., 2006), *Mucor circinelloides* (Fernández-Lahore et al., 1999), *Rhizopus oryaze* (Kumar et al., 2005), *Aspergillus oryzae* (Vishwanatha et al., 2009), *Endothia parasitica* (Sardinas, 1968), *Penecillium oxalicum* (Hashem, 1999), *Amylomyces rouxii* (Yu and Chou, 2005) and *Fusarium subglutinans* (Ghareib et al., 2001). Among the several microorganisms studied, *Rhizomucor miehei* and *Rhizomucor pusillus* have gained industrial acceptance as producers of milk-clotting enzymes.

Commercial rennet preparations usually contain tertiary (or unspecific) proteolytic activities, which may further degrade curd proteins, leading to its dissolution and to the production of bitter peptides. Another striking feature that hinders the use of microbial rennets is their high thermal stability which allows them to extend their action on milk proteins after coagulation. Although after cheese making

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only 0 to 15% of the rennet activity added to milk is retained in curd (Sousa et al., 2001), the mentioned characteristics of commercial fungal rennets may cause extensive proteolysis resulting in low cheese yields and poor product quality. Particularly, soft and semi-hard cheese varieties are affected by extensive proteolytic activities (Hynes et al., 2001). Moreover, excessive proteolysis may degrade proteins of economic value that are present in the cheese whey.

A possible solution to alleviate the technical challenges described before is the identification of milk-clotting enzymes with enhanced specificity and reduced thermo tolerance. This may allow for limited proteolytic action and subsequent rapid destruction of the protease during further manufacturing operations e.g. heating. Fernández-Lahore et al. (1999) proved that aspartic proteases produced from certain mesophilic *Mucor sp. strain* (*M. bacilliformis* and *M. circinelloides*) have, like bovine chymosin, less heat stability than those from thermophilic fungi. However, the production of the aspartic proteinases from such microorganisms was only possible *via* solid state fermentation. On the other hand, *M. mucedo* has been screened as a mesophilic fungi possessing milk clotting activity (Fraile et al., 1978) and some of the factors affecting the production has been already reported by the use of local isolates (Handel and Fraile, 1984; Mashaly et al., 1981).

The aim of the present study was to investigate the growth conditions of *Mucor mucedo* DSM 809 in submerged fermentation to explore the production of milk-clotting enzymes and to obtain a better understanding on the biology of that microorganism during cultivation in shake flasks. Since other mesophilic *Mucor sp.* strains have been reported as producers of aspartic proteinases only under solid state fermentation conditions, *M. mucedo* can be a promising microorganism for milk-clotting enzyme production in submerged fermentation. The later is widespread in industry as a process that can be readily scaled-up for enzyme production.

MATERIALS AND METHODS

Microorganism and Culture Conditions

The microorganism used for the production of the milk-clotting enzyme was *Mucor mucedo* DSM 809, obtained from the German Collection of Microorganisms and Cell Cultures-DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). The strain was maintained by subculturing on potato-dextrose agar (PDA) slants at 24°C. Spores were harvested after 5 days of surface cultivation by suspension in sterilized distilled water. Inoculum size was adjusted by manual spore counting in a Neubauer chamber.

Growth Medium for submerged fermentation

To study effect of pH, carbon source, nitrogen source, shaking speed, and inoculum size experiments were carried out in duplicate Erlenmeyer flasks (250 ml) containing 30 ml of sterilized synthetic growth. The medium was inoculated with a spore suspension at the desired spore concentration.

The cultivation media consisted on a carbon source, a nitrogen source, and a mineral solution which were sterilized separately. The production media consisted in 1% (w/v) of the carbon source and 0.5 % (w/v) of the nitrogen source. Glucose, fructose, lactose and maltodextrin were tested as a carbon source while tryptone, casein and skim milk powder were tested as a nitrogen source. Casein concentration was further optimized. Carbon and nitrogen sources were autoclaved for 20 min at 120°C. The final concentration of each mineral in media is as follows (in g/L): $SO_4Mg.7H_2O$ 0.49; $SO_4Zn.7H_2O$ 0.0018; $SO_4Mn.H_2O$ 0.0003; $SO_4Cu.5H_2O$ 0.0004; $SO_4Fe.7H_2O$ 0.001; and KH_2PO_4 4.49. The mineral solution was prepared as a concentrate, adjusted to the final pH and sterilized by autoclaving (20 min at 121°C). 0.6 ml of this solution was added to 29.4 ml of the C/N production media in each of the flasks. Biomass was harvested by filtration and the filtrate was assayed for enzyme activity and other biochemical parameters.

Analytical procedures

Assay for milk clotting activity

Milk clotting activity was determined according to the method of Arima et al. (1970), which is based on the visual evaluation of the appearance of the first clotting flakes, and expressed in terms of Soxhlet units (SU). One Soxhlet unit is defined as the amount of enzyme that clots 1 ml of substrate in 40 min at 35°C. In order to perform the assay, 0.1 ml of the sample was added to a glass test tube containing 1 ml of reconstituted skim milk solution (10 g skim-milk powder dissolved in 100 ml of 0.01 M $CaCl_2$ solution) pre-incubated at 35°C for 10 min. The mixture was mixed well and the clotting time T (s) was measured with a chronometer. The clotting activity was calculated using the following formula:

$SU = (2400 \times 1 \times D)/0.1 \times T$
D: dilution of sample material
T: clotting time (s)

Assay for protease activity

Protease activity was determined by the azocasein digestion method, essentially according Samal et al. (1990). The reaction mixture contained 20 µl of azocasein substrate (5% w/v azocasein solution in 200mM Tris-HCl buffer, pH 7.5), 20 µl enzyme sample and 460 µl of 50 mM Tris HCl buffer, pH 7.5, were added. Following the incubation for 30 min at 37°C, 500 µl of TCA (10% w/v) was added and the samples were kept on ice 15 min. After centrifugation 7000 rpm for 10 min, 800 µl of supernatant was added to a tube containing 200 µl of NaOH (1.8 N). Absorbance was measured at 420 nm. The blank for each sample was prepared separately in the same manner except 500 µl of TCA was added before the addition of enzyme sample. One unit of protease activity is defined as the amount of enzyme that produces an increase in the absorbance of 0.003 under the above assay condition.

Protein determination

Protein was determined according to the Bradford procedure with bovine serum albumin as the standard (Bradford, 1976).

Carbohydrate determination

Total carbohydrate was determined employing the phenol-sulphuric-acid procedure *as per* Dubois et al. (1956). Glucose was utilized as

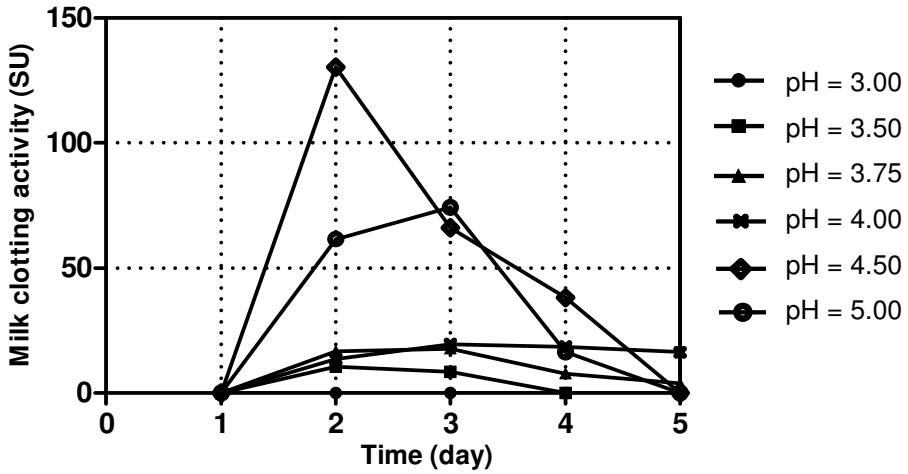

Figure 1. Effect of initial pH on milk clotting activity for 5 days fermentation period (Media: 1% glucose + 0.5% casein + 2% mineral solution; shaking speed: 220 rpm; cultivation temperature: 24°C; inoculum concentration: $2x10^4$ spore/ml; milk clotting activity for the samples having no activity after 2 hours incubation, was accepted as zero).

standard.

Biomass determination

For biomass determination, samples of the culture (30 ml) were clarified by filtration through a Whatman filter paper (Ø 90 mm). The separated solids (biomass) were dried until constant weight at 80 °C (Silveira et al., 2005).

RESULT AND DISCUSSION

Effect of initial pH

The pH of the medium is a very important but often neglected environmental factor. It can profoundly affect any biological activity being studied (Papagianni, 2004). In this study, pH was found to be a prevailing factor affecting milk-clotting enzyme production. The effect of initial pH of the growth medium on the enzyme activity during the course of a 5 days fermentation period is shown in Figure 1. The optimum initial pH value for maximum enzyme activity was found to be 4.50 (130 SU). No milk-clotting activity was detectable at pH 3.0 under similar cultivation conditions. It is evident that pH is a critical parameter influencing maximum production levels and enzyme biosynthesis kinetics. Mashaly et al. (1981) cultivated *M. mucedo* and found that the ratio milk clotting activity / proteolytic potency was higher when the initial pH value was adjusted to 3.75 while maximum milk-clotting enzyme production by *R. pusillus* (Ismail et al., 1984), *Amylomyces rouxii* (Yu and Chou, 2005), *Fusarium subglutinans* (Ghareib et al., 2001), and *Bacillus licheniformis* (D'Souza and Pereira, 1982) were reported to be maximum with an initial pH of 3.7, 7.0, 6.0 and 7.0 respectively. The optimal initial pH of the medium for milk-clotting enzyme production obviously may vary depending on the culture medium and microbial organism under study. Summarizing, the initial pH of the cultivation media is a parameter impacting on both maximum enzyme production levels and on the properties of the crude extract.

Effect of inoculum size

Three inoculums size values were tested: $2x10^3$, $2x10^4$, $2x10^5$ spores per ml of medium. The variation on inoculum size affected the biosynthesis of enzyme. A large inoculum decreased the level of enzyme obtained as observed by Fernández-Lahore et al. (1997). The maximum activity was obtained with a concentration of $2x10^4$ spores/ml, as is shown in Figure 2.

Effect of shaking speed

The production of the milk-clotting enzyme by *M. mucedo* DSM 809 as a function of shaking speed is shown in Figure 3. Among the shaking speed examined (220, 270, and 350 rpm), it was observed that milk-clotting enzyme production was highest at 220 rpm. Enzyme production decreased as the shaking speed increased, probably due to excessive shear stress which could damage biomass integrity. In contrast with Escobar and Barnett (1993), who claimed that the enzyme production was directly proportional to the shaking speed for *R. miehei*, the biosynthesis of the milk-clotting enzyme by *M. mucedo* DSM 809 decreased when the shaking speed increased above

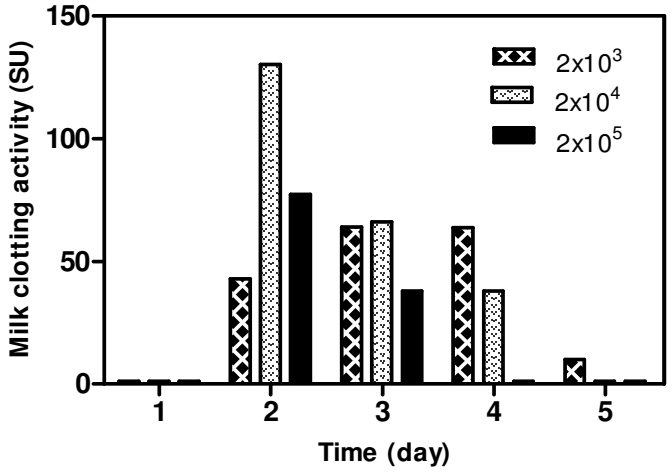

Figure 2. Effect of inoculum concentration on milk clotting activity for 5 days fermentation period (Media: 1% glucose + 0.5% casein + 2% mineral solution; shaking speed: 220 rpm; cultivation temperature: 24°C; initial pH of the medium 4.50; milk clotting activity for the samples having no activity after 2 hours incubation, was accepted as zero).

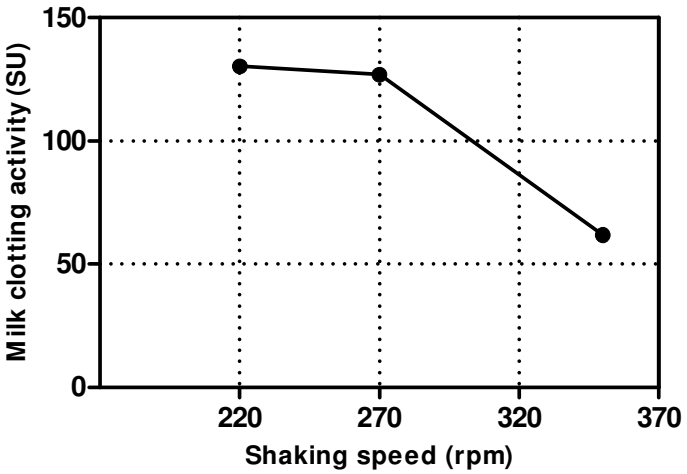

Figure 3. Effect of shaking speed on milk clotting activity at second day of fermentation (Media: 1% glucose + 0.5% casein + 2% mineral solution; cultivation temperature: 24°C; initial pH of the medium 4.50; inoculum concentration: $2x10^4$ spore/ml).

certain limits. The findings of the present study are similar to the observations made by Yu and Chou (2005) and Hashem (1999) who reported less milk-clotting activity with increased shaking speeds for *Amylomyces rouxii* and *Penicillium oxalicum,* respectively.

Effect of carbon source

To determine the most favorable source of carbon for enzyme production, either glucose, fructose, lactose, or maltodextrin were individually employed as a sole carbon source in the production medium. Figure 4 shows the activity levels obtained for each type of carbon source in the course of a 5 days fermentation period. Carbon sources stimulate rennet production depending on the metabolism of the microorganism under the study, as is the case of glucose for *R. miehei* fermentation (Silveira et al., 2005). Similarly, in the present study maximum enzyme activity was observed when glucose was used as a sole carbon source. On the contrary, no activity was obtained when lactose was employed. Channe and Shewale (1998) found that starch was a better carbon source for *A. niger* MC4 while Dutt et al. (2008) obtained maximum enzyme activity when fructose was used as a carbon source for *Bacillus subtilis*. Hashem (1999) found sucrose to be the most favorable carbon source for *Penicillium oxalicum*.

Effect of nitrogen source

Figure 5 depicts the influence of the nitrogen source on the biosynthesis of the milk-clotting enzyme by *M. mucedo* DSM 809. Among the nitrogen sources tested e.g. casein, tryptone, and skim-milk powder, the mentioned strain produced maximum enzyme levels in the presence of casein. On the other hand, the milk-clotting activity was the lowest when skim-milk powder was the sole organic nitrogen source. Silveira et al. (2005) showed that casein played an important role in rennin production under both solid state fermentation and submerged fermentation conditions in the case of *R. miehei*. According to Channe and Shewale (1998), casein triggered higher activity than tryptone and skim milk powder, when they were added separately to a fermentation media; the formation of milk clotting enzyme was very low when skim-milk powder was the sole source of nitrogen for *A. niger* MC4. Preetha and Boopathy (1994) observed that addition of skim-milk powder to the cultivation media did not improve the ratio of milk clotting activity to proteolytic activity during fermentation of *R. miehei*. Dutt et al. (2008) also tested several nitrogen sources for milk-clotting enzyme production by *Bacillus subtilis*. Their data indicate that addition of tryptone provided higher activity than the utilization of casein. From these studies it is clear that the optimal nitrogen sources vary considerably when comparing bacterial vs. fungal producers of milk-clotting enzymes.

Effect of casein concentration

As mentioned before casein proved to be an excellent substrate for the production of the milk-clotting enzyme from *M. mucedo* DSM 809. For this reason, casein levels were further optimized. Figure 6 shows the effect of casein concentration (0.5, 1.0, 1.5 and 2.0% w/v) on the enzyme

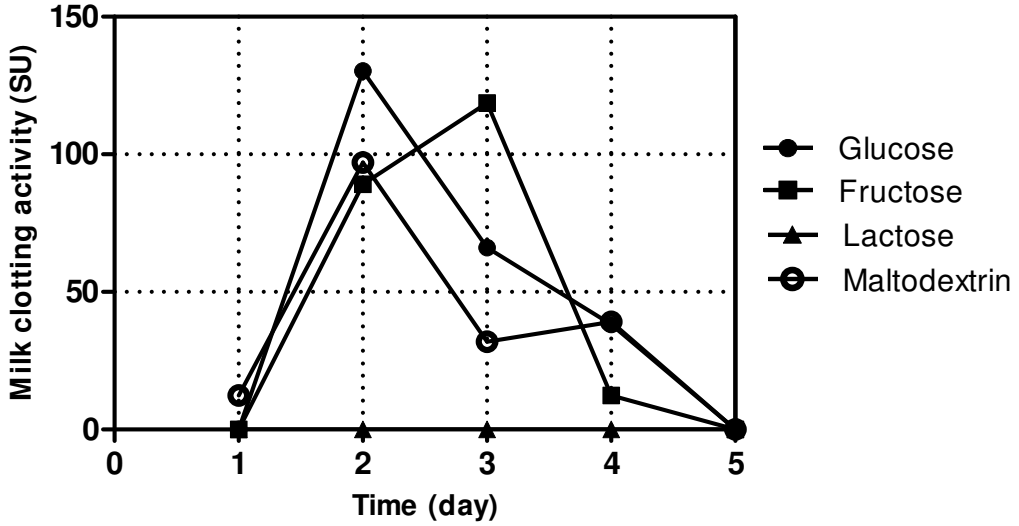

Figure 4. Effect of carbon source on milk clotting activity for 5 days fermentation period (Media: 1% carbon source + 0.5% casein + 2% mineral solution; shaking speed: 220 rpm; cultivation temperature: 24°C; initial pH of the medium 4.50; inoculum concentration: $2x10^4$ spore/ml; milk clotting activity for the samples having no activity after 2 hours incubation, was accepted as zero).

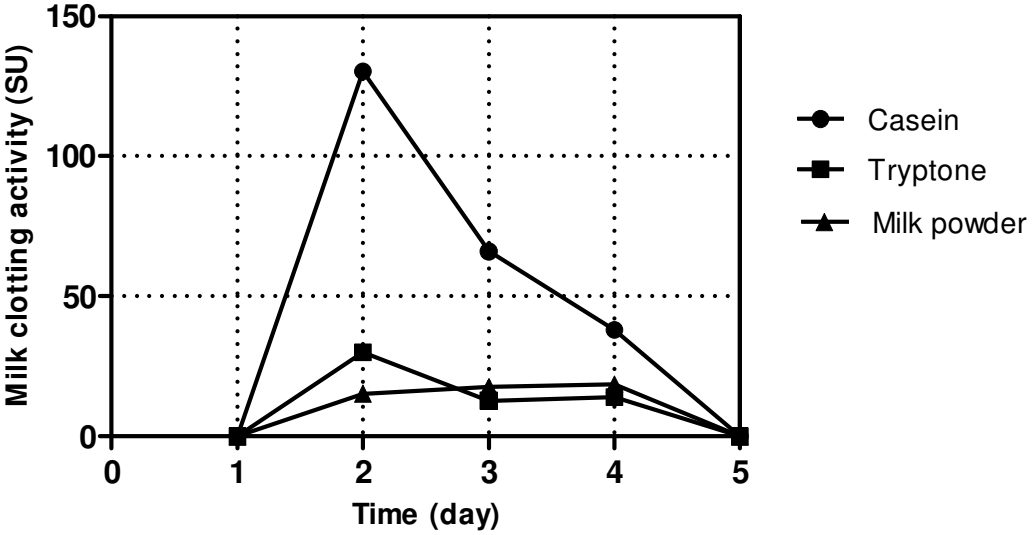

Figure 5. Effect of nitrogen source on milk clotting activity for 5 days fermentation period (Media: 1% glucose + 0.5% nitrogen source + 2% mineral solution; shaking speed: 220 rpm; cultivation temperature: 24°C; initial pH of the medium 4.50, inoculum concentration: $2x10^4$ spore/ml; milk clotting activity for the samples having no activity after 2 hours incubation, was accepted as zero).

production. The maximum milk-clotting activity was obtained when the casein concentration was set at 0.5% (w/v). Under such conditions, a peak of production was clearly observed after 48 h of cultivation. Similarly, Handel and Fraile (1984) also observed that *M. mucedo* produces maximum levels of a milk-clotting enzyme when the casein concentration in the cultivation media was set within the range 0.5 - 0.7% (w/v).

Culture profile and enzyme production

After modifying several nutritional and operational factors effecting the enzyme production during the course of the present study, a final cultivation profile was obtained for *M. mucedo* DSM 809. Table 1 shows the evolution of various cultivation parameters alongside the time of fermentation. It can be observed that the pH increased from 4.5 (at initial

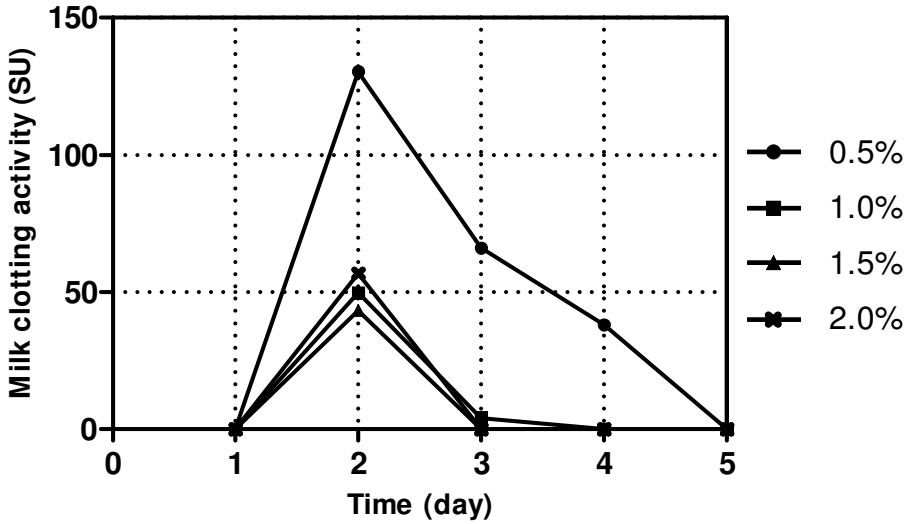

Figure 6. Effect of casein concentration on milk clotting activity for 5 days fermentation period (Media: 1% glucose + casein (0.5, 1.0, 1.5 and 2.0%) + 2% mineral solution; shaking speed: 220 rpm; cultivation temperature: 24°C; initial pH of the medium 4.50, inoculum concentration: $2x10^4$ spore/ml; milk clotting activity for the samples having no activity after 2 hours incubation, was accepted as zero).

Table 1. Final cultivation profile for *M. mucedo* DSM 809

Time (day)	pH	Milk clotting activity (SU)	Protease activity (PU)	MCA/PU[a]	Biomass (g/l)	Total CHO (g/l)	Total protein (µg/ml)
1	3.82±0.04	0	4.8±0.7	ND[b]	3.1±0.2	7.4±0.3	34.22±1.68
2	3.89±0.01	130.7±1.2	7.9±0.5	16.5	6.3±0.8	0.3±0.1	36.9±0.48
3	4.38±0.20	66.1±0.3	7.6±0.9	8.7	6.5±0.4	0.4±0.1	31.82±0.04
4	6.41±0.02	38.1±0.1	1.1±0.1	34.6	6.1±0.3	0.4±0.1	34.95±0.93
5	6.86±0.02	0	1.8±0.2	ND[b]	5.4±0.1	0.3±0.3	39.91±0.01

MCA/PU[a] = Milk clotting activity/Protease activity
ND[b] = Not determined

conditions) to 6.86 (after 5 days of cultivation). The maximum level of milk-clotting activity was observed at the second day of fermentation. Biomass levels increased from 3.1 g/l at the first day of cultivation to 6.5 g/l after 2-3 days of fermentation, to decrease afterwards. Biomass development was parallel to enzyme production, as it is expected for a primary product of metabolism. Total protease activity reached a maximum level (7.9 PU) on the second day of the fermentation as it was the case for the milk-clotting activity (130 SU). The total carbohydrate content decreased to negligible amounts at the second day of cultivation since it was consumed for biomass and enzyme synthesis.

Conclusion

The production of the milk-clotting enzyme by *M. mucedo* DSM 809 has been studied in submerged fermentation and the optimized cultivation profile was reported. The mentioned microbial strain could be a valuable source of an enzyme having a high milk-clotting to tertiary proteolysis ratio and decreased resistance to thermal treatment. *M. mucedo* is unique among other *Mucor* sp. strains in its ability to produce a milk-clotting enzyme in submerged fermentation as opposed to solid substrate cultivation. Further studies are needed in order to elucidate the nature and the characteristics of the enzyme(s) produced and to increase production levels in bioreactors.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Cytotoxic effect of betulinic acid and betulinic acid acetate isolated from *Melaleuca cajuput* on human myeloid leukemia (HL-60) cell line

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The cytotoxic effect of betulinic acid (BA), isolated from *Melaleuca cajuput* a Malaysian plant and its four synthetic derivatives were tested for their cytotoxicity in various cell line or peripheral blood mononuclear cells (PBMC) by 3-[4,5-dimethylthizol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Betulinic acid acetate (BAAC) was most effective than other betulinic acid derivatives. It had most active cytotoxic activity against human myeloid leukemia (HL-60), human T4-lymphoblastoid (CEM-SS), BALB/c murine myelomonocytic leukemia (WEHI-3B) and human cervical epithelial carcinoma (HeLa) but not on normal human lymphocytes (PBMC), suggesting its action is specific for tumor cells. BA and BAAC inhibit HL-60 cell line at low concentration after 72 h with IC_{50} values at 2.60 and 1.38 µg/mL, respectively. DNA fragmentation analysis showed ladder formation in the 100 - 1500 bp region in HL-60 cell lines after 24 h of treatment with IC_{50} values. The induction of apoptosis was also confirmed by flow cytometric analysis of cell cycle. BA and BAAC have been shown to induce a time dependant increase in the sub G_1 peak indicating apoptotic phenomenon as obtained from the DNA content histogram analysis. Thus, betulinic acid isolated from Malaysia plant showed good potential as an anti-cancer compound with less toxicity to human normal cells.

Key words: Betulinic acid, HL 60, cytotoxicity, MTT assay, DNA laddering, Cell cycle PI

INTRODUCTION

Betulinic acid (3β-hydroxy-lup-20(29)-ene-28-oic acid), an example of a pentacyclic triterpene is widely distributed in plant kingdom (Maurya et al., 1989). This compound can be chemically derived from betulin, a substance found in the outer bark of white birch tree *Betula alba* (Pisha et al., 1995). Some biological activities have been ascribed to betulinic acid, includes anti-inflammatory, anti-tumor (Mukherjee et al., 1997; Liu et al., 2004), anti-angiogenesis (Mukherjee et al., 2004), anti-viral (De Clercq, 1995; Baltina et al., 2003; Parlova et al., 2003), anti-HIV (Hashimoto et al., 1997; Huang et al., 2006; Qian et al., 2007), anti-neoplastic (Fulda et al., 1999) and anti-plasmodial (Ziegler et al., 2004).

Betulinic acid exerts a selective anti-tumor activity on cultured human melanoma (Pisha et al., 1995), neuroblastoma (Schmidt et al., 1997), malignant brain tumor

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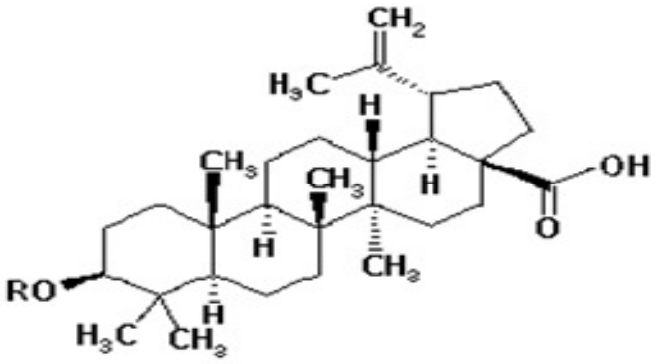

Compound	R
BA	H
BAAC	$COCH_3$
BAES	$COC(CH_3)_2CH_2COOH$
BASUC	$CO(CH_2)_2COOH$
BCL	COC_6H_5

Figure 1. Chemical structure of betulinic acid and its derivatives.

(Fulda et al., 1999) and leukemia cells (Hata et al., 2003). This compound showed inhibitory effect on leukemia (HL60, U937 and K562) and neuroblastoma (GOTO and NB-I) cell growth (Hata et al., 2003). It was reported selective against neuroblastoma cells and lacked side-effects. Moreover, its activity against neuroectodermal has been reported and related to the induction of apoptosis via direct mitochondrial alterations (Fulda et al., 1998). The anti-tumor activity of betulinic acid has been reported to other human neoplastic cell lines including lung, ovarian, cervical, colorectal, breast, and prostate cancer (Zuco et al., 2002; Kessler et al., 2007). Besides, Raghuvar Gopal et al. (2005) reported that betulinic acid had cytotoxic effect on human chronic myelogenous leukemia (CML) cells. Currently, betulinic acid is undergoing phase II clinical trials to treat melanoma (Gauthier et al., 2009). Betulinic acid was known to induce apoptosis selectively in tumor cells lines, but not on normal cell lines (Zuco et al., 2002). The favorable therapeutic index from the lack of toxicity towards normal cells suggested betulinic acid to be an attractive and promising anti-tumor agent (Pisha et al., 1995). This feature makes betulinic acid unique in comparison to compounds that are currently used in cancer therapy, such as taxol, camptothecin, elipticine, etoposide, vinblastine and vincristine. All these anti-tumor compounds are very toxic and inhibit replication of both cancer and normal cells (Zuco et al., 2002). In this project, the potential of betulinic acid isolated from Malaysian plant as anti-cancer agent on human myeloid leukemia will be investigated. Here, the *in vitro* cytotoxic activity of betulinic acid and its potent derivatives will be assessed using leukemic cell lines and compared with conventional anti-neoplastic drug (doxorubicin).

MATERIALS AND METHODS

Cell lines

The cancerous cell lines (anchorage-dependent and suspensions cells) were obtained from the American Type Culture Collection (ATCC), the National Cancer Institute (NCI) and the RIKEN Cell Bank (RCB). The suspensions cell lines were human myeloid leukemia (HL-60), human T4-lymphoblastoid (CEM-SS) and BALB/c murine myelomonocytic leukemia (WEHI-3B) whereas the anchorage-dependent cell lines were human cervical epithelial carcinoma (HeLa), human breast adenocarcinoma (MCF-7), human glioblastoma (DBTRG0.5MG) and mouse skin melanoma (B16).

The human myeloid leukemia (HL-60), human T4-lymphoblastoid (CEM-SS), BALB/c murine myelomonocytic leukemia (WEHI-3B), human cervical epithelial carcinoma (HeLa), human breast adenocarcinoma (MCF-7) and mouse skin melanoma (B16) were maintained in RPMI 1640 medium (Sigma, USA) supplemented with 10% foetal calf serum (GIBCO, UK) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) (GIBCO, UK). Human glioblastoma (DBTRG0.5MG) were maintained in RPMI 1640 medium (Sigma, Germany) supplement with 1% HT (Flowlab, Australia), 1% L-glutamine 200 mM (Flowlab, Australia), 0.5% sodium pyruvate 100 mM (Flowlab, Australia), 10% foetal calf serum (GIBCO, UK) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) (GIBCO, UK). All type of cells was incubated under 37°C, 5% CO_2.

Isolation of peripheral blood mononuclear cells (PBMC) from human peripheral blood

Blood samples were collected by venepuncture in 5 ml lithium heparin coated Vacutainers. Blood 20 to 25 mL was diluted 1:1 with phosphate buffered saline (PBS) and layered onto Ficoll-paque plus (Amersham) by using Pasteur pipette and centrifuged at 400 x g (1500 rpm) for 40 min at 18 to 20°C. The lymphocyte layer was transfered using a clean Pasteur pipette to a clean centrifuge tube and washed three times in balanced salt solution. The lymphocytes suspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum (GIBCO, UK) and antibiotics (100 units/ml penicillin and 100 µg/mL streptomycin) (GIBCO, UK). The lymphocytes were diluted to 1.0 x 10^6 cells/ml in DMEM and used immediately for MTT assay experiment.

Betulinic acid and its derivatives

Betulinic acid (BA), betulinic acid acetate (BAAC), 3-*O*-(2',2'-dimethylsuccinyl)-betulinic acid (BAES) and 3-*O*-succinyl-betulinic acid (BASUC) were kindly donated by Dr. Faujan B.H. Ahmad, while betulinic acid benzoate (BCL) was donated by Dr Yamin B. Yassin, both from the Department of Chemistry, Faculty of Science, University Putra Malaysia. Doxorubicin (DOX) was obtained from Sigma Aldrich, USA. These compounds (Figure 1) were used for the screening experiments however only betulinic acid, betulinic acid acetate and doxorubicin were used for further detail in cytotoxicity studies.

Betulinic acid

The pure compound of betulinic acid appears as a white solid, malted at 295 - 297°C. It was isolated from *Melaleuca cajuput* and was chromatographed on a silica gel column using chloroform as eluent (Ahmad et al., 1997).

Betulinic acid acetate

Betulinic acid acetate was prepared by refluxing betulinic acid (1.0 g) with acetic anhydride and pyridine in dichloromethane for several hours. After cooling to room temperature, water was then added and the white crystal was filtered off. Betulinic acid acetate appears as a pale yellow solid crystal. It also can be prepared by enzymatic reaction of betulinic acid and acetic anhydride in the present of lipase (Ahmad et al., 2005).

The compounds were dissolved in dimethylsulphoxide (DMSO) (Sigma, USA) at concentration of 10 mg/ml as a stock solution. It was then diluted further to a concentration of 60.0 µg/ml in RPMI 1640 by diluting 18.0 µl of the stock solution into 2982.0 µl RPMI 1640 as a working stock. The stock solution and working stock were both stored at 4°C.

MTT cytotoxic assay

MTT colorimetric assay was developed for measuring cell survival and proliferation. The principle behind this assay is that the tetrazolium ring in MTT is cleaved by dehydrogenases present in active mitochondria, resulting in the formation of an insoluble MTT formazan product (Mosmann, 1983). Briefly, 100 µl of RPMI-1640 or DMEM media with 10% of FBS was added into all the wells except row A in the 96 well plate (TPP, Switzerland). Then, 100 µl of diluted compound at 60 µg/ml was added into row A and row B. A series of two fold dilution of extract was carried out down from row B until row G. The row H was left untouched and the excess solution (100 µl) was discarded and 100 µl of cell line or PBMC with cell concentration at 1 X 10^5 cells/ml was added into all wells in the 96 well plate and incubated in 37°C, 5% CO_2 and 90% humidity incubator for selected period (24, 48, 72 h). After the corresponding period (either 24, 48 or 72 h), 20 µl of MTT (Sigma, USA) at 5 mg/ml was added into each well in the 96 well plate and incubated for four hours in 37°C, 5% CO_2 and 90% humidity incubator. Then, the plate was centrifuged at 200 x g for 5 min and 170 µl of medium with MTT was removed from every well. 100 µl DMSO (Fisher Scientific, UK) was added to each well to extract and solubilize the formazan crystal by incubating for 20 min in 37°C, 5% CO_2 incubator. Finally, the plate was read at 570 nm wavelength by using µ Quant ELISA Reader (Bio-Tek Instruments, USA). The results of the compounds were compared with the result of Doxorubicin and without drug by using the same method. Each compound and control was assayed in triplicate for three times. The percentage of proliferation was calculated by the following formula: Proliferation (%) = [(OD sample – OD control) / OD control] X 100.

DNA fragmentation assay

The HL-60 cells at concentration 1 x 10^5 cells/ml were treated with doxorubicin, betulinic acid and betulinic acid acetate with IC_{50} and 30 µg/ml concentration in 6 well plates. The triplicate samples were incubated for 24, 48 and 72 h at 37°C, 5% CO_2, 90% humidity. The treated cells were collected by centrifugation at 300 x g for 10 second and the DNA was isolated from cells for each treatment using The Wizard® Genomic DNA Purification Kit (Promega, USA). After harvesting, the cells were washed with 200 µl phosphate buffer saline (PBS). The cell pellet was then resuspended in 600 µl Nuclei Lysis Solution in the presence of 3 µl RNase and incubated for 30 min at 37°C. Then, the sample was allowed at room temperatures for 5 min. After 5 min, 200 µl of Protein Precipitation Solution was added to sample and vortex for 20 s. The sample chilled on ice for 5 min and centrifuged at 13000 x g for 4 min. The supernatant was removed to clean microcentrifuge tube contain 600 µl isopropanol and centrifuged at 13000 x g for 1 min. After decanting the supernatant, 600 µl 70% ethanol was added and centrifuged at 13000 x g for 1 min. Then, the ethanol was aspirated using pipette and dried the pellet for 15 min. 100 µl of DNA Rehydration Solution and incubated for 1 h at 65°C. The DNA was stored at 4°C.

Agarose gel (1%) was prepared. The gel was then submerged into running buffer (TBE buffer). Subsequently, 8 µl of DNA mixed with 2 µl loading dye (80% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol) was loaded on 1% agarose gel. Samples were loaded into the wells and were run at 40 V for 12 h. The agarose gel was then stained with ethidium bromide (Sigma, USA) (0.5 µg/ml in running buffer) for 30 min and destained with distilled water for an hour. DNA was visualized by UV illuminator (245 nm).

Flow cytometric propidium iodide cell cycle analysis

The HL-60 cells at concentration 1 x 10^6 cells/ml were treated with doxorubicin, betulinic acid and betulinic acid acetate with IC_{50} values in 6 well plates. The triplicate samples were incubated for 24, 48 and 72 h at 37°C, 5% CO_2, 90% humidity. The treated cells were collected by centrifugation at 2000 rpm for 10 min and the pellet was fixed in 500 µl ice-cold ethanol 70% at -20°C for 2 h. Then, the cell suspension was centrifuged, washed twice with 1 ml of PBS solution containing 0.06% sodium azide and resuspended in 1 ml of PBS solution containing 0.1% triton X-100, 100 mM EDTA, 50 µg/ml RNase (Sigma, USA) and 2 µg/ml propodium iodide (PI) (Sigma, USA). The tubes were placed on ice in the dark until the red fluorescence of DNA-bound PI in individual cells was measured by Beckman Epics Ultra flow cytometer and the results were analyzed using Expo32 software (Beckman Coulter, USA).

RESULTS

MTT cytotoxic effect of betulinic acid, betulinic acid derivatives and doxorubicin on various cancer cell lines

The cytotoxic effect of betulinic acid (BA), betulinic acid acetate (BAAC), 3-*O*-(2',2'-dimethylsuccinyl)-betulinic acid (BAES), 3-*O*-succinyl-betulinic acid (BASUC), betulinic acid benzoate (BCL) and doxorubicin (DOX) obtained against human myeloid leukemia (HL-60), human T4-lymphoblastoid (CEM-SS), murine myelomonocytic leukemia (WEHI-3B), human cervical epithelial carcinoma (HeLa), human breast adenocarcinoma (MCF-7), human glioblastoma (DBTRG0.5MG) and mouse skin melanoma (B16) cell lines was determined by a rapid colorimetric assay, using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT).

The cytotoxic activity of BA and its derivatives were tested using different cell lines and the cytotoxicity data of these compounds on HL-60 (human myeloid leukemia), CEM-SS (human T4-lymphoblastoid), WEHI-3B (BALB/c murine myelomonocytic leukemia), HeLa (human cervical epithelial carcinoma), MCF-7 (human breast adenocarcinoma), DBTRG0.5MG (human glioblastoma) and B16 (mouse skin melanoma) were summarized in Table 1.

The cytotoxic effects of betulinic acid and its derivatives were examined in HL-60 leukemia cell line after 72 h treatment. The IC_{50} values for betulinic acid (BA),

Table 1. The cytotoxicity data of betulinic acid (BA) and betulinic acid derivatives (BAAC, BAES, BASUC and BCL) against various cell lines. The inhibition concentration of 50% (IC_{50}) value was measured by MTT assay after 24, 48 and 72 h treatment.

Cell line	Time (h)	IC_{50} value (µg/ml)				
		BA	BAAC	BAES	BASUC	BCL
HL-60	24	20.70 ± 5.39	15.10 ± 2.57	>30.0	>30.0	>30.0
	48	14.60 ± 2.31	4.20 ± 1.67	18.70 ± 6.47	2.00 ± 1.00	>30.0
	72	2.60 ± 1.50	3.50 ± 1.03	1.38 ± 0.50	1.45 ± 1.06	2.10 ± 1.01
CEM-SS	24	5.40 ± 1.06	9.20 ± 2.19	>30.0	>30.0	>30.0
	48	2.30 ± 1.14	8.90 ± 3.55	>30.0	>30.0	>30.0
	72	2.10 ± 0.52	3.60 ± 0.53	>30.0	1.60 ± 0.81	>30.0
WEHI-3B	24	18.90 ± 1.65	13.70 ± 1.21	>30.0	>30.0	>30.0
	48	15.50 ± 6.87	16.60 ± 3.65	>30.0	>30.0	>30.0
	72	2.10 ± 1.03	3.90 ± 2.45	22.0 ± 2.16	>30.0	>30.0
HeLa	24	>30.0	>30.0	>30.0	>30.0	>30.0
	48	16.8 ± 0.60	3.0 ± 1.10	22.7 ± 0.42	27.4 ± 1.83	>30.0
	72	2.5 ± 3.70	3.1 ± 0.92	10.5 ± 2.53	22.8 ± 3.55	>30.0
MCF-7	24	>30.0	>30.0	>30.0	>30.0	>30.0
	48	>30.0	>30.0	>30.0	>30.0	>30.0
	72	20.4 ± 2.91	10.5 ± 1.03	20.3 ± 2.14	>30.0	10.4 ± 1.05
DBTRG0.5MG	24	>30.0	>30.0	>30.0	>30.0	>30.0
	48	>30.0	>30.0	25.9 ± 2.97	>30.0	>30.0
	72	>30.0	>30.0	10.1 ± 1.87	>30.0	>30.0
B16	24	>30.0	>30.0	>30.0	>30.0	>30.0
	48	>30.0	>30.0	>30.0	>30.0	>30.0
	72	>30.0	>30.0	>30.0	>30.0	>30.0

betulinic acid acetate (BAAC), 3-*O*-(2',2'-dimethyl-succinyl)-betulinic acid (BAES), 3-*O*-succinyl-betulinic acid (BASUC) and betulinic acid benzoate (BCL) against HL-60 cell lines were 2.60 ± 1.50, 3.50 ± 1.03, 1.38 ± 0.50, 1.45 ± 1.06 and 2.10 ± 1.01 µg/ml, respectively.

Amongst the leukemia cell lines, BAAC was the most toxic than other betulinic acid derivatives after 24 h treatment on HL-60, CEM-SS and WEHI-3B cell lines with the IC_{50} values of 15.10 ± 2.57, 9.20 ± 2.19 and 13.70 ± 1.21 µg/ml, respectively. Higher IC_{50} values were observed when BAES, BASUC and BCL were tested against HL-60, CEM-SS and WEHI-3B after 24 hours with IC_{50} values more than 30.00 µg/mL for all the cell lines. The sensitivity of HL-60, CEM-SS and WEHI-3B cell lines treated with BAAC at 72 h were almost similar resulting in the IC_{50} value of 3.50 ± 1.03, 3.60 ± 0.53 and 3.90 ± 2.45 µg/ml, respectively.

The results indicate that both BA and BAAC showed cytotoxic activity against HL-60, CEM-SS and WEHI-3B cell lines after 24 h treatment. The IC_{50} values of BA against HL-60, CEM-SS and WEHI-3B were 20.70 ± 5.39, 5.40 ± 1.06 and 18.90 ± 1.65 while the IC_{50} values for BAAC were 15.10 ± 2.57, 9.20 ± 2.19 and 13.70 ±1.21 µg/ml, respectively. BA showed strong cytotoxicity against HL-60, CEM-SS and WEHI-3B after 72 h treatment with IC_{50} value of 2.60 ± 1.50, 2.10 ± 0.52 and 2.10 ± 1.03 µg/ml, respectively.

In HeLa cancer cell line, BA was the best compound for cytotoxic activity followed by BAAC, BAES and BASUC. The results showed that both BA and BAAC exhibited the most cytotoxic to HeLa cell line after 72 h treatment with the IC_{50} values of 2.50 ± 3.70 and 3.10 ± 0.92 µg/ml, respectively. BAES showed the low cytotoxic effect against HeLa and DBTRG0.5MG after 72 h treatment with the IC_{50} value of 10.5 ± 2.53 and 10.1 ± 1.87 µg/ml, respectively.

In MCF-7 cancer cell line, BA and its derivatives showed less cytotoxic activity with IC_{50} values more than 10.00 µg/mL, each. The IC_{50} values of HL-60 after treatment with BA, BAAC, BAES and BCL for 72 h were 20.4 ± 2.91, 10.5 ± 1.03, 20.3 ± 2.14 and 10.4 ± 1.05 µg/ml, respectively. Betulinic acid and all its derivatives did not show a significant cytotoxicity against DBTRG0. 5MG and B16 cell lines with their IC_{50} values were more than 30.00 µg/ml. The screening results for cytotoxic activity showed that HL-60 was the most sensitive cell line towards betulinic acid and its derivatives after 72 h treatment.

Betulinic acid and its derivatives were also screened for cytotoxicity on other cancer cell lines such as the

Table 2. The cytotoxicity data of doxorubicin (DOX) betulinic acid (BA) and betulinic acid acetate (BAAC) against human myeloid leukemia (HL-60) and normal human lymphocytes (PBMCs). The inhibition concentration of 50% (IC_{50}) value was measured by MTT assay after 24, 48 and 72 h.

Cell line	Time (h)	IC_{50} value (µg/ml)		
		DOX	BA	BAAC
HL-60	24	0.56 ± 0.25	20.7 ± 5.39	15.1 ± 2.57
	48	0.43 ± 0.06	14.6 ± 2.31	4.2 ± 1.67
	72	0.21 ± 0.03	2.6 ± 1.50	3.5 ± 1.03
PBMC	24	>30.0	>30.0	>30.0
	48	>30.0	>30.0	>30.0
	72	>30.0	>30.0	>30.0

cytotoxicity data of BA and its derivatives on HeLa, MCF-7, DBTRG0.5MG and B16 cell lines were summarized in Table 1.

Since BAAC exhibited strong cytotoxicity against all leukemia cells, it was selected as the test compound in further study. Table 2 showed the cytotoxicity data of DOX, BA and BAAC against human myeloid leukemia (HL-60) and normal human lymphocytes (PBMC) after 24, 48 and 72 h treatment. DOX a known commercial drug was used as a positive control in this study.

In HL-60 cell line, DOX appeared to be the most toxic drug with the IC_{50} values of 0.56 ± 0.25, 0.43 ± 0.06 and 0.21 ± 0.03 µg/ml at 24, 48 and 72 h of treatment, respectively. BAAC was more cytotoxic at 24 and 48 h treatment compared with BA with IC_{50} values of 15.10 ± 2.57, 4.20 ± 1.67 and 20.70 ± 5.39, 14.60 ± 2.31 µg/ml, respectively. In the 72 h treatment, BAAC was less cytotoxic than BA with IC_{50} values of 3.5 ± 1.03 and 2.60 ± 1.50 µg/ml, respectively.

The IC_{50} values of DOX, BA and BAAC on HL-60 were also compared with PBMC cell lines. Table 2 showed the results that DOX, BA and BAAC were not toxic to normal human lymphocytes (PBMC) with IC_{50} values more than 30.00 µg/ml, each.

DNA fragmentation effect of doxorubicin, betulinic acid and betulinic acid acetate on HL-60 cell lines

The DNA fragmentation, a biochemical hallmark of apoptosis was detected by DNA fragmentation assay. Detection of DNA fragmentation is currently one of the most frequently used techniques in the study of cell death. Internucleosomal DNA fragmentation can be visualized by gel electrophoresis as the characteristic DNA ladder pattern. DNA from apoptotic cells display a ladder formation by 1% agarose gel electrophoresis analysis of DNA extracted from HL-60 cells treated with IC_{50} values of doxorubicin (DOX), betulinic acid (BA) and betulinic acid acetate (BAAC).

DNA fragmentation was observed at 24, 48 and 72 h after exposure to 0.2 µg/ml doxorubicin (Figure 2a). Cells treated with 2.6 µg/ml betulinic acid (Figure 2b) and 3.5 µg/ml betulinic acid acetate (Figure 2c), demonstrated a ladder pattern of DNA fragments were slightly detectable after 24 h and became visible after 48 and 72 h exposured. The patterns could not be detected in untreated HL-60 cells as the negative control (lane 1). The internucleosomal DNA fragmentation was confirmed by the pattern of DNA laddering into fragments with multiples of 180 - 220 base pairs detected in agarose gel electrophoresis of extracts obtained at 24 h from HL-60 treated with DOX, BA and BAAC.

Flow cytometry analysis of doxorubicin, betulinic acid and betulinic acid acetate on HL-60 cell lines

The cells with hypodiploid DNA were analyzed by Beckman Epics Ultra flow cytometer after PI staining at 24, 48 and 72 h treatment with IC_{50} values of doxorubicin (DOX), betulinic acid (BA) and betulinic acid acetate (BAAC) to confirm the state of apoptosis. The percentage of cells with hypodiploid (sub G_1), which represent the fraction undergoing apoptotic DNA degradation that appeared in the cell distribution with DNA content less than G_1 was measured.

The cell cycle distribution of treated HL-60 cell lines in both BA and BAAC was almost similar with a sub G_1 of apoptosis population. The number of apoptotic cells in HL-60 cells increased slightly at this stage after 24, 48 and 72 h of treatment with BA and BAAC. When exposed to 2.6 µg/ml BA for 24, 48 and 72 h the apoptotic cells was demonstrated 8.75, 9.90 and 12.21% (Table 3) respectively. Approximately 6.20, 8.37 and 13.35% (Table 3) of apoptotic cells after 24, 48 and 72 h of treatment with 3.5 µg/ml of BAAC.

The DNA content of HL-60 cells treated with IC_{50} values of doxorubicin (DOX), betulinic acid (BA) and betulinic acid acetate (BAAC) was determined using flow cytometer. The distribution of DNA content was expressed as sub G_1, G_1/G_0, S and G_2/M phase, inclusively to see if there was any arrest of the growth of the treated cells. The IC_{50} concentration of DOX, BA and BAAC was used to assess the extent of the arrest of cell growth after 72 h of treatment. Table 3 shows the cell cycle

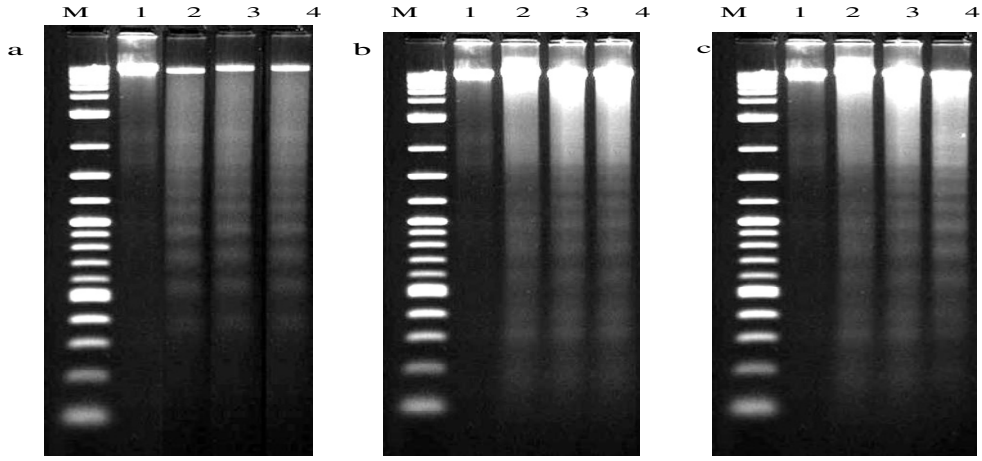

Figure 2. DNA ladder formation following exposure of HL-60 cells to doxorubicin, betulinic acid and betulinic acid acetate. (a) DNA fragmentation induced by untreated HL-60 cells (lane 1), HL-60 cells treated with 0.2 µg/ml doxorubicin for 24, 48 and 72 h (lane 2 - 4), (b) DNA fragmentation induced by untreated HL-60 cells (lane 1), HL-60 cells treated with 2.6 µg/ml betulinic acid for 24, 48 and 72 h (lane 2 - 4) and (c) DNA fragmentation induced by untreated HL-60 cells (lane 1), HL-60 cells treated with 3.5 µg/ml betulinic acid acetate for 24, 48 and 72 h (lane 2 - 4). M is 1 kb ladder.

Table 3. The cell cycle distribution of doxorubicin (DOX), betulinic acid (BA) and betulinic acid acetate (BAAC) in HL-60 cell lines. HL-60 cells were treated with 0.2 µg/ml of DOX, 2.6 µg/ml of BA and 3.5 µg/ml of BAAC at 24, 48 and 72 h.

Treatment		Cell cycle (% of total cells)			
		Sub G_1	G_1/G_0	S	G_2/M
DOX (0.2 µg/ml)	Untreated	1.08	48.30	18.29	32.33
	24 h	5.92	51.68	21.85	20.55
	48 h	16.85	24.47	43.55	15.13
	72 h	60.11	7.62	27.28	4.99
BA (2.6 µg/ml)	Untreated	1.08	48.30	18.29	32.33
	24 h	8.75	21.78	42.54	26.93
	48 h	9.90	32.77	38.35	18.98
	72 h	12.21	43.67	23.15	20.97
BAAC (3.5 µg/ml)	Untreated	1.08	48.30	18.29	32.33
	24 h	6.20	39.99	37.92	15.89
	48 h	8.37	27.78	46.36	17.49
	72 ho	13.35	33.56	40.49	12.60

distribution of HL-60 cells following exposure to 0.2 µg/ml of DOX, 2.6 µg/ml of BA and 3.5 µg/ml of BAAC in the sub G_1, G_1/G_0, S and G_2/M phase. The cell cycle distribution after 24, 48 and 72 h of treatment were compared with untreated control cells. The distribution of untreated HL-60 cells represented 1.08% in sub G_1 population, 48.30% in G_1/G_0 population, 18.29% in S population and 32.33% in G_2/M population. The majority of cells were in G_1/G_0 phase in untreated HL-60 cells with 48.30% of cell population. The sub G_1 cell population was increased with an accompanying significant decrease in the G_2/M population after 24, 48 and 72 h of treatment with DOX, BA and BAAC.

The cell cycle profile in Table 3 showed an increase in the proportion of cells in S phase with 42.54% of cells and decrease in the proportion of cells in G_1/G_0 and G_2/M with 21.78 and 26.93% of cells after 24 h of treatment with 2.6 µg/ml of BA. The HL-60 cells have undergone

arrest at S phase with 42.54 and 38.35% of cells after 24 and 48 h followed by arrest at G_1/G_0 phase with 43.67% of cells after 72 h. As a result, cells were unable to enter the subsequent G_2/M phase with 20.97% cells compared to 26.93% at 24 h. The percentage of cells population in G_2/M phase was reduced to 20.97% compared to 32.33% of untreated HL-60 after 72 h showed that BA also inhibit the proliferation of HL-60 cells.

On the other hand, BAAC at 3.5 µg/ml also induce G_1/G_0 arrest after 24 h of treatment. The cell cycle distributions in G_1/G_0 phase were 39.99, 27.78 and 33.56% after 24, 48 and 72 h of treatment, respectively. BAAC induced a significant increase in the proportion of cells in S phase after 48 h. The percent of cells in this phase became depreciate from 46.36% at 48 h to 40.49% at 72 h after treatment. At 72 h of treatment with BAAC the S phase arrested cells appeared to be capable of entering the following G_2/M phase, accounting for 12.60% cells compared to 17.49% at 48 h. The cell cycle blockage progress from G_1/G_0 to S and G_2/M phase with increasing the incubation time. Collectively, the data show that in HL-60 cells, the IC_{50} value of BAAC produced a cell population in which some cells were undergoing S phase cell cycle arrest while others were undergoing DNA degradation.

DISCUSSION

Cytotoxic has been defined as the cell killing property of a chemical compound independent from the mechanism of death (Graham-Evans et al., 2003). Assessment of a compound's toxicity to various cell types can be made using *in vitro* cytotoxicity tests, which are available and widely used. The inclusion of an *in vitro* cytotoxicity assay in early discovery efforts provides an important advantage in identifying potentially cytotoxic compounds (Hamid et al., 2004). One effect of reactive chemicals potentially encountered at subtoxic concentrations is the direct interaction with DNA that will result in various types of damage, including promutagenic lesions (Eisenbrand et al., 2002).

Cytotoxicity data are of their own intrinsic value in defining toxic effects (e.g. as an indicator of acute toxic effects *in vivo*) and are also important for designing more in-depth *in vitro* studies (Eisenbrand et al., 2002). The effective dose for a 50% reduction in cell number for plants products to be considered cytotoxic should be less than 20 µg/ml (Geran et al., 1972). The IC_{50} which is the drug concentration that kill 50% of the cells was determined graphically after 24, 48 and 72 h of treatment with doxorubicin (DOX), betulinic acid (BA) and betulinic acid derivatives on human myeloid leukemia (HL-60), human T4-lymphoblastoid (CEM-SS), BALB/c murine myelomonocytic leukemia (WEHI-3B), human glioblastoma (DBTRG0.5MG), mouse skin melanoma (B16) and human peripheral blood mononuclear cells (PBMC) and used as a measure of cytotoxic effect.

The cytotoxic effects of four betulinic acid derivatives compounds (BAAC, BAES, BASUC and BCL) were examined in seven cancer cell lines. These BA and its derivatives are the lupane triterpene with a carbonyl group at C-17 that has been modified at C-3 hydroxy group. In screening studies, several derivatives have shown better cytotoxicity than BA. Betulinic acid acetate (BAAC) had shown broad spectrum cytotoxicity than other betulinic acid derivatives. It had most active cytotoxic activity against human myeloid leukemia (HL-60), human T4-lymphoblastoid (CEM-SS), BALB/c murine myelomonocytic leukemia (WEHI-3B) and human cervical epithelial carcinoma (HeLa) but not on normal human lymphocytes (PBMC).

It is known that the cytotoxicity of isoprenoid carboxylic acid derivatives is often related to the presence of a free carboxyl group in the molecule (Mutai et al., 2004). In general, C-28 carboxylic acid group in betulinic acid and its derivatives was found essential for providing cytotoxic activity (Muherjee et al., 2004). Furthermore the position of the hydroxyl on C-3 is more important than on C-28, the presence of the conjugated carbonyl influences the activity very slightly and finally the presence of two hydroxyls (on C-3 and C-28) results in a reduction of activity (Mutai et al., 2004).

The study of the relationships between structure and activity of lupane triterpenes demonstrated that the carbonyl group at C-17 played an important role on the induction of melanoma cell apoptosis (Hata et al., 2003) Lup-28-al-20(29)-en-3-one, betulinic acid (BA) and other lupane triterpene with a carbonyl group at C-17 showed marked cytotoxic effects on leukemia, neuroblastoma and melanoma cells, but not on normal lung fibroblast cells (W138). It seemed that the carbonyl group at C-17 might be essential for the induction of cancer cell apoptosis by these triterpenes (Hata et al., 2003).

The modification structure at C-3 hydroxy group of BA produced potentially BAAC which may develop as antitumor drugs. The relationships between the structure and activities of BA and BAAC on the induction of HL-60 cell differentiation and apoptosis were studies. This study focused on HL-60 cells, a human leukemia cell line that readily undergoes apoptosis in response to a variety of chemotherapeutic agents (Kaufmann, 1989). HL-60 cell has been widely used as a model for studying pre-myelocytic cell differentiation and the identification of differentiation-inducing agents (Poon et al., 2004).

In this study, BA isolated from *M. cajuput* a Malaysian plant and all its derivatives have indicated significant growth inhibition in HL-60 human leukemia cancer cell line at low concentration of IC_{50} values. BA from this *M. cajuput* marked the IC_{50} values at 2.60 µg/ml (5.70 µM). This result is same as the experiment that was done using BA from Aldrich (St Louis, MO) at the concentration range of 1 to 12 µM (0.47 to 5.47 µg/ml) that showed toxicity to HL-60 cells with IC_{50} value at 5.7 µM and

induced apoptosis in about 10% of surviving cells. Part of the toxic effect may be due to its inhibitory effects on topoisomerase I and II in some cell types and therefore may affect DNA replication (Chowdhury et al., 2003). In other study, it was reported that the IC_{50} values of BA against the cell growth of HL-60 was 6.6 µM (Hata et al., 2003).

One of the interesting findings was that DOX, BA and BAAC showed very high IC_{50} values towards normal PBMC cell lines. The IC_{50} values of DOX, BA and BAAC were up to 30 µg/ml. It has been reported that BA from Sigma Chemical, St. Louis, MO, USA also showed the IC_{50} values up to 50 µg/ml on the peripheral blood lymphoblast (PBL) whereas the IC_{50} of DOX was 0.020 ± 0.002 µg/ml. Thus on normal PBL BA was at least 1000 fold less toxic than DOX (Zuco et al., 2002). This differential cytotoxicity of BA and these compounds towards the normal and cancerous cell, could be taken advantage of in therapeutics.

It has been reported that the low concentrations of several different drugs in HL-60 cells induced cell death by apoptosis while higher concentration caused necrotic cell death when cells were assessed morphologically and by DNA gel electrophoresis (Lennon et al., 1991). Low concentration of BA and BAAC took a longer time for the degradation of large fragments of DNA to smallest fragments of approximately 200 bp. This kind of ladder formation was because DNA fragmentation during apoptosis proceeds through an ordered series of stages beginning with the production of DNA fragments of 300 kbp, which are then degraded to fragments of 50 kbp. Fragments of this size are further degraded to smaller fragments of 10 to 40 kbp and finally to small oligonucleosome fragments of 180 to 200 bp that are recognized as the characteristic DNA ladder. These phenomena could be further confirmed using pulsed-field gel electrophoresis (PGFE), which can detect the initial stage of DNA fragmentation to fragments of 300 and 50 kbp (Walker et al., 1993).

The biochemical hallmark of apoptosis is the orderly 200 base pairs DNA ladder fragmentations, which have recently been further characterized as the signature of apoptosis from apoptosis specific endonucleolytic cleavages (Zhang and Xu, 2002). The apoptosis signature cleavages are also suggested as the cause of 50 kilobase (kbp) high molecular weight fragmentations that mark early or stage 1 apoptosis (Zhang et al., 2000), but the association appears to be not an imperative correlation (Samejima et al., 2001). 50 kbp fragmentations are known to be expressed in necrosis which has not been shown expressing neither 200 bp ladder fragmentations nor specific megabase level fragmentations (Bicknell and Cohen, 1995).

In many systems, DNA fragmentation has been shown to results from activation of an endogenous Ca^{2+} and Mg^{2+} dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units, a linker DNA generating mononucleosomal and oligonucleosomal DNA fragments. These DNA fragments reveal, upon agarose gel electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180 base pairs subunit. This is the biochemical hallmark of apoptosis with the fragmentation of the genomic DNA, an irreversible event that commits the cell to die (Cohen, 1993).

Flow cytometry allows a simultaneous estimation of cell cycle parameters and apoptosis. There is compelling evidence that apoptotic death induced by chemopreventive or chemotherapeutic agents is closely linked to perturbation of a specific phase of the cell cycle. The effect of a given antiproliferative agent on cell cycle progression appears to depend on the concentration of the compound and also on the duration of the treatment (Surh et al., 1999). The induction of apoptosis was also confirmed by flow cytometric analysis of cell cycle. Doxorubicin (DOX), betulinic acid (BA) and betulinic acid acetate (BAAC) have been shown to induce a time dependant increase in the sub G_1 peak with the decrease of cells in diploid regions (G_1, G_2 and S-phase) indicating apoptotic phenomenon as obtained from the DNA content histogram analysis.

Cell cycle analysis revealed that BA induced apoptosis and cell cycle arrest at G_0/G_1 phase in HL-60 cell. Approximately 8.75% of viable cell were in the sub G_1 indicating the apoptotic phase was found after treatment with 2.6 µg/ml of BA for 72 h. The G_0/G_1 arrest shown by the above compounds therefore, suggest that these agents may slow down the growth of cancer cells by artificially imposing the cell cycle checkpoint. Among these checkpoints, p53 is the most vital G1 checkpoint protein that can either lead to growth arrest in G1 or apoptosis (Dou et al., 1995).

This finding was similar with the previous report that apoptosis towards HL-60 cell with the IC_{50} value of 5.7 µM induced apoptosis in cell cycle analysis with approximately 10% of viable cells in the sub G_1 phase after exposure of the cell to 12 µM of BA for 72 h treatment (Poon et al., 2004). However, the apoptosis rate of BA towards HL-60 is considered low if compared to its derivatives 23-hydroxybetulinic acid. 23-hydroxybetulinic acid induced apoptosis in HL-60 cells with approximately 46.61% cells were in sub G_1 after exposure of cells to 10 µM for 24 h. Subsequently, the apoptotic events in their experiment were associated with concurrent down-regulation of Bcl-2 and telomerase activity (Ji et al., 2002).

It has been widely reported that DHD_3 initially increases cell proliferation, which is followed by cell differentiation and maturation (Brown et al., 1999). DHD_3 at 1 µM altered cell cycle distribution with an increased G_1 population after 72 h of incubation. The addition of various concentrations of BA (3 to 6 µM) to 1 µM DHD_3 resulted in a does-dependent and statistically significant increase in the G_1 population with a concomitant

reduction in the S phase population. Less than 2% of cells were apoptotic under these conditions (Poon et al., 2004). Arrest in S and G2/M phase as strongly evident in etoposide-treated population may be contributed by extensive chromosome damage (Arita et al., 1997). Principally, it acts by inhibiting topoisomerase II that entangles excessive twists or knots in the DNA helix which would otherwise arise during replication. This enzyme makes a transient double strand break in the first duplex to create DNA 'gate' for the nearby second duplex to pass through and then reseals the break (Berger et al., 1996).

Differentiated cells are normally inactive in cell division and arrested in G_1 phase of the cell cycle. Significant G_1 arrest was observed when HL-60 cells were treated with DHD_3. This G_1 arresting action of DHD_3 was also enhanced with the addition of BA, but cell number was not affected under the same conditions (Poon et al., 2004). DHD_3 initially accelerates cell proliferation, which is followed by cell differentiation and maturation (Brown et al., 1999). A single DHD_3-treated HL-60 cell would give rise to 10 or more matured monocytes. The action of BA in enhancing DHD_3 induced NBT reduction, membrane marker expression and G_1 cell cycle arrest provide corroborative evidence that BA and DHD_3 act synergistically in inducing differentiation in HL-60 cells (Poon et al., 2004).

Cell cycle arrest is one of the targets of many anticancer drugs, such as doxorubicin, cisplatin, 5-fluorouracil and paclitaxel. It has been shown that the ability of cells to arrest cell cycle in G_2/M or S phase was related to their drug sensitivity and increased with cell resistance (Dubrez et al., 1995). Induction of apoptosis and/or cell proliferation inhibition is highly correlated with the activation of a variety of intracellular signaling pathways to arrest the cell cycle in the G1, S, or G2 phase. In malignant tumors cell, population in G_1 phase appear less frequent (< 70%) than in normal tissue (> 90%). The damages that cause G_1-check point arrest are believed to be irreversible process and the cells ultimately undergo apoptosis (Roy et al., 2004).

In summary, betulinic acid (BA) and betulinic acid acetate (BAAC) showed selective cytotoxic towards all HL-60, CEM-SS and WEHI-3B leukemic cell line and are not toxic to PBMC. This study demonstrated that those compounds are potentially good anti cancer drug since they are non-toxic towards healthy cell.

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Full Length Research Paper

Chemical control of blossom blight disease of sarpagandha caused by *Colletotrichum capsici*

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Sarpagandha (*Rauvolfia serpentina* Benth) is grown in different parts of India and its adjoining countries for its root which is the chief source of several important alkaloids like ajmalicine, ajmaline, isoajmaline, rauvolfinine, reserpine and serpentine. Blossom blight caused by *Colletotrichum capsici* is one of the most serious diseases of sarpagandha in the North Indian plains. Eight fungicides, namely Benomyl, Carbendazim, Chlorothalonil, Mancozeb, Metalaxyl-mancozeb, Propiconazole, Thiram and Thiophanate-methyl were evaluated against the spore germination and mycelial growth of *Colletotrichum capsici* under *in vitro* condition. The results indicated that Mancozeb, Metalaxyl-mancozeb, Propiconazole and Thiram inhibited percent spore germination at the dose of 2 - 10 µg/ml, while Benomyl, Carbendazim and Thiophanate-Methyl were ineffective even at 250 µg/ml concentration. Propiconazole, Carbendazim, Benomyl, Mancozeb and Metalaxyl-mancozeb were highly effective inhibiting 50% mycelial growth at 2.8, 4.6, 6.0, 9.3 and 11.2 µg/ml, respectively, while Thiophanate-methyl, Chlorothalonil and Thiram were relatively less effective showing 50% mycelial growth inhibition at 25.2, 27.2 and 31.3 µg/ml, respectively. The effective fungicides were further employed for the protection of sarpagandha from blossom blight disease in the field during the year 2006 and 2007. The results of the two years (2006 and 2007) of field experiments indicated that disease incidence was significantly reduced in all the treated plots over unsprayed control. The spray of Mancozeb and Carbendazim produced highest protection that is >80% in both the years against blossom blight disease and their treatments also produced healthy and highest seed yield per plant.

Key words: Blossom blight, *Colletotrichum capsici*, fungicides, *Rauvolfia serpentina*, sarpagandha.

INTRODUCTION

Sarpagandha (*Rauvolfia serpentina* Benth .Ex.Kurz family Apocynaceae) is growing in different parts of India and its adjoining countries. Its root contains many important alkaloids, like ajmalicine, ajmaline, isoajmaline, rauvolfinine, reserpine, serpentine, rescinnamine, tetra-phylicine, yohimbine and 3 epi α-yohimbine (Snimolia et al., 1984). Sarpagandha has been used in several Ayurvedic formulations from ancient times. Its extract served as an antidote for the bites of poisonous reptiles and insects, and also used as hypotonic and sedative in various disorders, in anxiety states, excitement, maniacal behavior associated with psychoses, insanity, insomnia and epilepsy. Blossom blight caused by *Colletotricum capsici* is one of the most important diseases of sarpagandha in North Indian plains and caused drastic reduction in the production of healthy seeds (Shukla et al., 2006). The disease incidence adversely affects the sarpagandha plantation because of severe infection on inflorescence leading to premature death of the infected flowers. The blossom blight also resulted in decapitation and prevented seed setting.

There are many reports on the chemical control of the diseases caused by the pathogen, *C. capsici* on other hosts (Mishra, 1988; Eswaramurthy et al., 1988; Ekbote, 2005) but the work on chemical control of blossom blight disease of sarpagandha has not been carried out so far. Thus, attempts have been made to evaluate the efficacy of different fungicides against the pathogen, *C. capsici in-vitro* and apply effective fungicides for two consecutive

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Table 1. Minimum inhibition concentration (MIC) and EC-50 values of different fungicides against spore germination and mycelial growth of *Colletotrichum capsici*.

Fungicides	Spore germination		Mycelial growth	
	MIC (µg/ml)	EC-50 (µg/ml)	MIC (µg/ml)	EC-50 (µg/ml)
Benomyl	> 250**	-	> 5 < 100	6.0
Carbendazim	> 250	-	> 5 < 100	4.6
Chlorothalonil	> 5 <50	27.5	> 5 < 250	27.2
Mancozeb	> 3 <10	3.5	> 5 < 100	9.3
Metalaxyl-mancozeb	> 3 <10	4.2	> 5 < 100	11.2
Propiconazole	> 2 < 10	2.8	> 2 < 10	2.8
Thiram	> 3 < 10	3.0	> 5 < 250	31.3
Thiophanate-methyl	> 250	-	> 5 < 250	25.2

*All the *in vitro* effects were calculated based on data from five Petri dishes.
**Supported spore germination but inhibited mycelial growth even at very low dose.

years for the protection of sarpagandha from blossom blight disease in the field and results are reported.

MATERIALS AND METHODS

Fungicides

Benomyl (Benlate 50% WP, E.I DuPont India Ltd, New Delhi-17, India), Carbendazim (Bavistin 50% WP, BASF India Ltd, Thane-Belapur Road, Thane, Mumbai, India), Chlorothalonil (Kavach 75% WP, Syngenta India Ltd., Mumbai-20, India), Mancozeb (Dithane M-45, 75% WP, Indofil Industries Ltd., Bari Brahamana, Jammu, India), Metalaxyl-mancozeb (Ridomil MZ 72% WP, Syngenta India Ltd., Mumbai-20, India), Propiconazole (Tilt 25% EC, Syngenta India Ltd., Mumbai-20, India), Thiram (Thirox 75% WS, Crop Chemicals Ltd., Ernakulam, Cochin-031,India) and Thiophanate–methyl (Topsin–M 70% WP, Motilal Pesticides Pvt Ltd., Masani, Mathura-003, India) were used to test their activity *in-vitro* and effective ones were applied in the field to control blossom blight disease of sarpagandha.

Evaluation against spore germination and mycelial growth of *C. capsici*

For testing spore germination inhibition, spore of *C. capsici* (1x106 spores ml-1) were treated by different concentrations (5 to 250 ug/ml) of fungicide solution. The spores treated with sterile distilled water served as control. One ml treated as well as non-treated (control) spore suspension was spread over thin poured 2% water agar medium in 5 replicated Petri dishes (90 mm) and incubated at 23 ± 2°C. All the tests and treatment were repeated twice. Percentage of spore germination was recorded after 16 h and percentage of spore germination inhibition over control was determined in each of the treatments.

Based on active ingredients, the required quantity of different fungicides was incorporated individually into melted potato-dextrose-agar (PDA) to obtain 5, 10, 50, 100 and 250 µg/ml concentration. The medium without fungicide served as control. Aliquot of 20 ml of each fungicide added medium was poured into 90 mm Petri dishes with 5 replicates. They were later inoculated by 6 mm diameter. mycelial disc from 7 day- old- culture of *C. capsici* and incubated at 23 ± 2°C. Mycelial growth inhibition was determined after 6 days and EC-50 (effective concentration for inhibiting 50% radial growth) values were determined by the method of Bliss (1934) and Vincent (1947).

Effective chemical control of blossom blight disease of sarpagandha in the field

Five fungicides showing effective performance under *in-vitro* evaluation were used in different doses. The experiment was carried out for the two consecutive years (2006 and 2007) in complete randomized block design with plot size 3 x 2 m^2 at CIMAP experimental field, Lucknow. The soil type of the experimental field was sandy loam; standard package of practices for *R. serpentina* recommended for North Indian plains were followed during the course of study (Khanuja et al., 2005). Sarpagandha plantations were carried out in the field at a distance of 45 x 60 cm in the month of February 2005 - 2006. All the treatments were performed in triplicate. First spray was given just after appearance of initial symptoms of the disease on the flowers in second week of July. Thereafter; 3 more applications were done at the interval of 15 days till 2nd week of September. Tween-20 (Hi-Media Laboratories Pvt. Ltd Mumbai India) at 0.25 ml/l was added to the fungicide solution as a spreader/sticker during rainy season. The effectiveness of fungicides was evaluated 15 days after the fourth application by rating infection on the inflorescence of the infected plants in each of the plots on a 0 - 4 scale (Shukla et al., 2006). The percent disease index (PDI) and percent disease control (PDC) was calculated by the formulae evolved by McKinney (1923) and Chester (1950), respectively. The data were analyzed statistically and critical difference (CD) was calculated at 5%.

RESULTS

Effect of fungicides on the spore germination and mycelial growth of *C. capsici*

Results shown in Table 1 indicated that minimum inhibition concentrations (MICs) of the fungicides against spore germination were invariably less than those against the radial mycelial growth. Propiconazole was most effective as it inhibited 91.1% spore germination of *C. capsici,*

Table 2. Chemical control of sarpagandha blossom blight (%) and seed yield/plant in the field.

Fungicides	Doses (%)	2006			2007		
		Disease incidence (%)	Disease control (%)	Average seed yield* g/plant	Disease incidence (%)	Disease control (%)	Average seed yield* g/plant
Control	_	52.84	_	1.08	54.97	-	1.37
Benomyl	0.15	13.63	74.21	5..56	15.02	72.64	8.87
Carbendazim	0.15	8.00	84.86	8.89	9.02	83.59	10.15
Mancozeb	0.30	6.43	87.83	11.67	8.78	84.03	13.61
Metalaxil-mancozeb	0.15	11.75	77.67	6.48	13.71	75.06	8.45
Propiconazoe	0.15	21.53	59.25	4.14	_	_	_
S Em	_	1.48	_	0.51	1.23	_	0.54
CD. at 5%	_	4.67	_	1.60	3.87	_	1.69

*Average seed yield was calculated from 5 plants with 3 replicates.

followed by Thiram (64.1%), Mancozeb (60.9%), and Metalaxly-mancozeb (58.1%) at 5 µg/ml, while Benomyl, Carbendazim and Thiophanate-methyl were ineffective as their treatment failed to inhibit spore germination even at 250 µg/ml concentration. Propiconazole, Carbendazim, Benomyl, Mancozeb and Metalaxyl-mancozeb inhibited 50% mycelial growth at 2.8, 4.6, 6.0, 9.30 and 11.3 µg/ml, respectively, while Ec-50 values for Chlorothalonil, Thiram and Thiophanate-methyl were 27.2, 31.3 and 25.2 µg/ml, respectively.

Effective chemical control of blossom blight disease of sarpagandha in the field

During the year 2006, four sprayings with each of five selected fungicides (Table 2) over the flowers and foliage of the sarpagandha plants in the field caused significant reduction disease incidence over untreated control. Mancozeb and Carbendazim were most effective as their treatment produced 87.83 and 84.86% protection, respectively over untreated control. Metalaxyl-mancozeb and Benomyl were the next effective fungicides producing 77.67 and 74.21% protection but the effect was not significantly different in between these two fungicides. Propiconozole was least effective producing 59.25% disease control. For this reason it was not used in the year 2007.

Results of the year 2007 are shown in Table 2.where four fungicides, namely Benomyl, Carbendazim, Mancozeb and Metalaxyl-mancozeb were applied in the field. Mancozeb and Carbendazim produced almost similar results as in 2006. They exhibited 8.78 and 9.02% disease incidences and highest protection that is 84.03 and 83.59%, respectively over control (Figure 1). The disease incidences in control plots were recorded to be 52.84 and 54.97% in 2006 and 2007, respectively. The protection due to fungicide application was significant, although the efficacy of different fungicides differ from each other. Our experimental results (Table 2) also indicated that seed yield/plant was increased significantly over control due to fungicidal spray. The spray of Mancozeb and Carbendazim in the year 2006 and 2007 invariably produced maximum seed yield/plant that is 11.67-13.61 g and 8.89 - 10.15 g, respectively. In the unsprayed control plots, plants either produced very little amount of seeds/plant or seed setting was not at all present.

DISCUSSION

The *in vitro* effect of fungicides did not match with their performances *in vivo* against blossom blight disease of sarpagandha. For instance, Carbendazim did not have high effect against mycelial growth and spore germination of the pathogen at lower concentrations but its field performance was effective and significant. It has inhibited conidial germination of *C. capsici* at higher concentrations thereby produced effective control of the disease in the field. Mancozeb was highly effective against conidial germination at lower concentrations but not against the mycelial growth. It gave promising protection from *C. capsici* infections in the field (Figure 1). The control of anthracnose and fruit rot disease of chili caused by *C. capsici* was achieved by spraying with Carbendazim and Mancozeb (Das and Mohanty, 1988; Biswas, 1992; Ebenezar and Alice, 1996). Sometimes, fungicide highly effective *in vitro* was not effective *in vivo*, as we observed with Propiconazole. It was highly effective against spore germination and mycelial growth of *C. capsici* but was ineffective in the field when sprayed over the flowers and foliage. Such finding was also reported by Margina and Zheljazkov (1994). It is attributed that this may be due to non-absorption/decomposition by the plant.

Figure 1. Effective control of blossom blight disease of sarpagandha with spraying by Mancozeb under field condition.

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Full Length Research Paper

Key region of laminin receptor 1 for interaction with human period 1

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The 67 kDa laminin receptor 1 (Lamr1) is a novel protein that interacts with human circadian clock protein period 1 (hPer1). We confirmed the interaction between hPer1 and complete Lamr1 (295 amino acids) through yeast two-hybrid system in the present study. And we identified the interaction between hPer1 and hLamr11-190/hLamr1201-295 with yeast two-hybrid system. The results showed that hPer1 could interact with two partial Lamr1, which each contained a laminin-binding region, suggesting that both two partial sequences contained the binding region for hPer1. To define the key region of Lamr1 to interact with hPer1, pGADT7-Rec/hLamr11-190 was mutated with the palindromic sequence LMWWML, part LMW and WML, respectively. With yeast two-hybrid system, we found that hPer1 could not interact with Lamr1 mutated with LMWWML and LMW, but could interact with Lamr1 mutated with WML. It suggested that the palindromic sequence LMWWML in peptide G of Lamr1, especially LMW of it, was necessary for the interaction. Although, the palindromic sequence LMWWML is just the actual binding site for laminin. Together, these findings suggested that hPer1 might interact with Lamr1 by occupying the laminin-binding sites. It will be beneficial for studying the mechanism of hPer1 interaction with Lamr1.

Key words: Laminin receptor 1 (Lamr1), human circadian clock protein period 1 (hPer1), interaction, yeast two-hybrid, key region.

INTRODUCTION

Circadian clocks are molecular time-keeping mechanisms that reside in a diverse range of cell types in a variety of organisms. The primary role of these cell-autonomous clocks is to maintain their own approximately 24 h molecular rhythms and to drive the rhythmic expression of genes involved in physiology, metabolism and behavior (Andretic and Hirsh, 2000; Dunlap, 1999). In mammals, a an autoregulatory transcriptional-translational-feedback loop involving a set of clock genes. master circadian clock resides in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. The SCN is composed of multiple, single-cell circadian oscillators, which, when synchronized, lead to coordinated circadian outputs that ultimately regulate overt rhythms (Reppert, 2000). On the molecular level, the oscillator consists of

Central components of the mammalian circadian clock are the period (Per) genes which are expressed in a diurnal manner in various tissues on the RNA and protein levels (Field et al., 2000; Tei et al., 1997). Human Period 1 (hPer1), expressed widely in brain regions, non-neuronal tissues and cell lines, not only regulates the circadian oscillator but also plays some additional roles, such as drug dependence and tumor development (Andretic et al., 1999; Liu et al., 2005; Filipski et al., 2002). As a member of the basic helix-loop-helix (HLH)/

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Abbreviations: SCN, Suprachiasmatic nucleus; **hPer1,** human circadian clock protein period 1; **Lamr1,** laminin receptor 1.

Per/Arnt/Sim (PAS) family of proteins, hPer1 also can form multiple heterodimers and their available sub-unit could directly influence many multiple pathways. Therefore, according to Per1's complex functions, many people speculate that there were some other proteins that interact with Per1. Previous study finds that the 67 kDa laminin receptor 1 (Lamr1) is a novel protein that interacts with human circadian clock protein hPer1 and Lamr1 is not a direct efferent element of circadian clock (Wang et al., 2007). But how does hPer1 interact with Lamr1? We still have not known the key region of Lamr1 to interact with hPer1.

The 67 kDa Lamr1, a membrane-bound mature form of 37 kDa laminin receptor precursor (37LRP), is present on the cell surface and functions as a membrane receptor for the adhesive basement membrane protein laminin (Hundt et al., 2001; Menard et al., 1998). The 67 kDa receptor subunit and its precursor appear to play an important role in several physiologic as well as pathologic processes, including cell differentiation, growth, migration and cancer invasion (Berno et al., 2005; Faury, 1998; Orihuela et al., 2009; Qiu et al., 2008). Peptide G (20 amino acids, IPCNNKGAHSVGLMWWMLAR), according to the amino acid residues 161-180 of Lamr1, binds specifically with high affinity to laminin (Castronovo et al., 1991; Magnifico et al., 1996; Taraboletti et al.,1993). Another binding site is at the carboxyl terminal (RDPEEIEKEEQAAAEKAVTKEEFQG, amino acids 205 - 229) (Gloe et al., 1999; Landowski et al., 1995). Wang et al. (2007) find that the sequence is encoded amino acids from 56 to 295 of *Homo sapiens* Lamr1 including the sequence which is shown to bind laminin. We suspected that hPer1 might interact with Lamr1 by occupying the laminin-binding sites. Because most functions of Per1 were based on protein-protein interaction, we utilized the classical techniques, yeast two-hybrid system and site-directed mutagenesis, to define the key region of Lamr1 to interact with hPer1.

MATERIALS AND METHODS

Cell culture

A549 cells (Human lung adenocarcinoma epithelial cell line) were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) supplemented with antibiotics (BioWhitaker, USA) and 10% fetal calf serum (Hyclone, USA) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Isolation of the specific DNA fragments of hLamr1

Total RNA of cells was isolated with Trisol reagent (Invitrogen, USA). The specific DNA fragments of hLamr1 were cloned from cellular total RNA by reverse transcription-polymerase chain reaction (RT-PCR) according to the RNA PCR kit manual (TaKaRa, Japan) using hLamr1 gene specific primers based on the hLamr1 cDNA sequence (gi: 59859884). The aimed sequences were encoded amino acids from 1 to 295 (Complete sequence), 1 to 190 and 201 to 295 of hLamr1, respectively. The primer sequences were 5' - GTACATATGATGTCCGGAGCCCTTGAT - 3' and 5' - CG CCAGTCTAGATTAAGACCAGTCAGTG - 3' for hLamr11-295; 5' - GTACATATGATGTCCGGAGCCCTTGAT - 3' and 5' - GCATCT AGAGGAAATGGTGCCACGC - 3' for hLamr11-190; 5' - GCGGCA TATGCTGTACTTCTACAGAGAT - 3' and 5' - CGCCAGTCTAGAT TAAGACCAGTCAGTG - 3' for hLamr1201-295. To construct the pGADT7-Rec/hLamr11-295, pGADT7-Rec/ hLamr11-190 and pGADT7-Rec/hLamr1201-295 plasmids, the specific amplified DNA sequences of hLamr1 were subcloned into pGADT7-Rec prey vectors (BD Biosciences Clontech, USA) which contained the GAL4 activation domain (AD) and their sequences were analyzed, respectively.

Screening partial Lamr1 interacting with hPer1 with yeast two-hybrid system

To initially investigate the key region of Lamr1 to interact with hPer1, we adopted yeast two-hybrid system. The different parts of hLamr1 were cloned in-frame in the yeast expression vector pGADT7-Rec, respectively. Previously the bait plasmid pGBKT7/ hPer1132 - 497 has been successfully constructed by Wang et al. in our lab (Wang et al., 2007). The HLH-PAS domain of hPer1 cDNA was cloned in-frame in the yeast expression vector pGBKT7, which contained the GAL4 DNA-binding domain (DNA-BD). Then the constructed vector pGBKT7 was introduced into the yeast strain AH109 by lithium acetate transformation. Transformed yeasts were selected on tryptophan-deficient medium. To identify the interaction between hPER1 and LAMR1, we adopted the Matchmaker Library Construction and Screening Kits (BD Biosci-ences Clontech, USA).

The prey plasmids pGADT7-Rec/hLamr11-295, pGADT7-Rec/ hLamr11-190 and pGADT7-Rec/hLamr1201-295 were transformed into the yeast strain AH109 which contained the bait plasmid pGBKT7/hPer1132-497. Because the prey vector pGADT7-Rec carried the leucine gene, it was conferred to have the capacity to grow in leucine-deficient medium. In addition, when bait and prey fusion proteins interact in AH109 yeast reporter strain, the DNA-BD and AD are brought into proximity and activate transcription of ADE2 and HIS3 reporter genes. These two genes allowed the yeast to grow in histidine-deficient medium and adenine-deficient medium. The positive clones were selected by -Ade/-His/-Leu/-Trp dropout (DO) supplement yeast selection medium (BD Biosciences Clontech, USA).

Colony-lift filter β-galactosidase assay

Besides ADE2 and HIS3 genes, lacZ is also a typical reporter gene in AH109 yeast strain to identify protein-protein interaction. And it could create a blue stain in the presence of 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal). X-gal must be used as the β-galactosidase substrate for the colony-lift filter β-galactosidase assay. Transformants to be assayed were lifted onto a Nitrocellulose filter (Pall Gelman, USA). Then the filter was treated with at least one freeze/thaw cycle in liquid nitrogen to lyse the yeast cell walls. Freeze-thaw cycles are a rapid and effective cell lysis method which permits accurate quantification of β-galactosidase activity. Carefully place the filter, colony side up, on the filter presoaked with Z buffer/X-gal solution (Sigma-Aldrich, USA). Incubate the filters at 30°C (or room temperature) and check periodically for the appearance of blue colonies.

Site-directed mutations in partial Lamr1

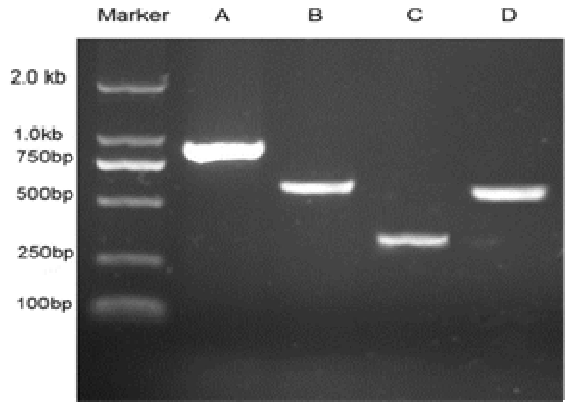

Figure 1. Expression of hLamr1 with RT-PCR. Complete sequence and two partial sequences targeting Homo sapiens Lamr1 were cloned from total RNA of A549 cells by RT-PCR. The electropherogram showed the expression of three kinds of Lamr1 and the housekeeping gene GAPDH. GAPDH was used as the internal control. Maker: DL2000 DNA Marker; A: Lamr11-295; B: Lamr11-190; C: Lamr11-295; D: GAPDH.

To further investigate the key region of Lamr1 to interact with hPer1, several site-directed mutations were induced in pGADT7-Rec/hLamr11-190. Because of too many mutated sites, we selected two suitable single restriction enzyme cutting sites Dra Ш and Xba I (New England Biolabs, USA), and then cut the partial sequence which contained the amino acids to be mutated from pGADT7-Rec/hLamr11-190 plasmid with the two sites. While the different DNA sequences which contained the different mutations were synthesized and ligated to the cut plasmid DNA ligation Kit Ver.2.1 (TaKaRa, Japan). The ligation products were transformed into *Escherichia coli* DH5a host cells, respectively. Three kinds of mutant plasmids were obtained: a. AD/Lamr1-LL: the mutation of LMWWML (amino acid residues 173 -178); b. AD/Lamr1-LMW: themutation of LMW (amino acid residues 173 - 175); c. AD/Lamr1-WML: the mutation of the WML (amino acid residues of 176 - 178). The sequences of these mutant plasmids were analyzed. The mutated sites are listed below: 173Aa TTA (Leucine, L) →TTC (Phenylalanine, F); 174Aa ATG (Methionine, M)→ACG (Threonine, T); 175Aa TGG(Tryptophan, W)→TCG (Serine, S); 176Aa TGG(Tryptophan, W)→TGC (Cysteine, C); 177Aa ATG (Methionine, M) →ACG 530bp(Threonine, T); 178 CTG (Leucine, L) →CCG (Proline, P).

Screening key region of Lamr1 to interact with hPer1 and yeast two-hybrid system

The interaction between hPer1 and mutant Lamr1 was identified by yeast two-hybrid system. Three kinds of mutant plasmids AD/Lamr1-LL, AD/Lamr1-LMW and AD/Lamr1-WML were transformed into the yeast strain AH109 which contained the bait plasmid pGBKT7/hPer1132–497. The positive clones with reporter genes ADE2, HIS3 and lacZ were selected by -Ade/-His/-Leu/-Trp dropout (DO) supplement yeast selection medium and colony-lift filter β-galactosidase assay.

RESULTS

Confirmation of the interaction between hPer1 and Lamr1

To further confirm the interaction between hPer1PAS and Lamr1, complete sequence of hLamr1 encoded by 295 amino acids was cloned from total RNA of A549 cells by RT-PCR (Figure 1A). The prey plasmid pGADT7-Rec/hLamr11-295 was constructed successfully. DNA sequence analyses showed that the DNA sequence of the insert fragment was completely homologous with that of Homo sapiens Lamr1 (gi: 59859884). The interaction between hPer1 and Lamr1 was further confirmed with yeast two-hybrid system. Clones containing bHLH-PAS domain of hPer1 and hLamr1 could be seen in SD (synthetic dropout) /-Leu/-Trp and SD-Ade/-His/-Leu/-Trp selection mediums (Figure 2a). The blue stain was also obviously observed in colony-lift filter β-galactosidase assay (Figure 3a). These results confirmed that Lamr1 could interact with hPer1 definitely.

Identification of two partial Lamr1 interacting with hPer1

To initially investigate the key region of Lamr1 to interact with hPer1, two partial DNA fragments of hLamr1, each containing one interaction region between laminin and Lamr1, were isolated from total RNA of A549 cells by RT-PCR (Figure 1). The specific DNA sequences of hLamr1 were subcloned into pGADT7-Rec prey vectors and their sequences were analyzed. The amino acid sequences of the two partial hLamr1 were identical with that of hLamr1 (amino acid residue: 1-190 and 201-295). With yeast two-hybrid system, both clones contained hPer1 PAS and hLamr11-190 and clones contained hPer1PAS and hLamr1201-295 could grow in SD/-Leu/-Trp and SD-Ade/-His/-Leu/-Trp selection mediums (Figure 2). And the colony-lift filter β-galactosidase assay also showed the same results. The results revealed that both two partial Lamr1 could interact with hPer1.

Definition of the key region of Lamr1 to interact with hPer1

To further study the key region of Lamr1 to interact with hPer1, three kinds of different site-directed mutated pGADT7-Rec/hLamr11-190 plasmids were constructed. Their sequences were analyzed. All the mutated sites were just the mutations we needed, and other sequences were identical with the origin. The palindromic sequence LMWWML (amino acid residues 173 -178) of hLamr1 was mutated to FTSCTP in AD/Lamr1-LL, LMW (amino acid residues 173 -175) were mutated to FTS in AD/Lamr1-LMW, and WML (amino acid residues 176 -178) were mutated to CTP in AD/Lamr1-WML (Figure 4).

The interaction between hPer1 and mutant Lamr11-190 were identified with yeast two-hybrid system. The results showed that the clones contained hPer1PAS and Lamr1-

LL also hPer1 and Lamr1-LMW which could grow in SD/ -Leu/-Trp selection mediums, but not in SD-Ade/-His/-Leu/-Trp mediums. However, the clones contained

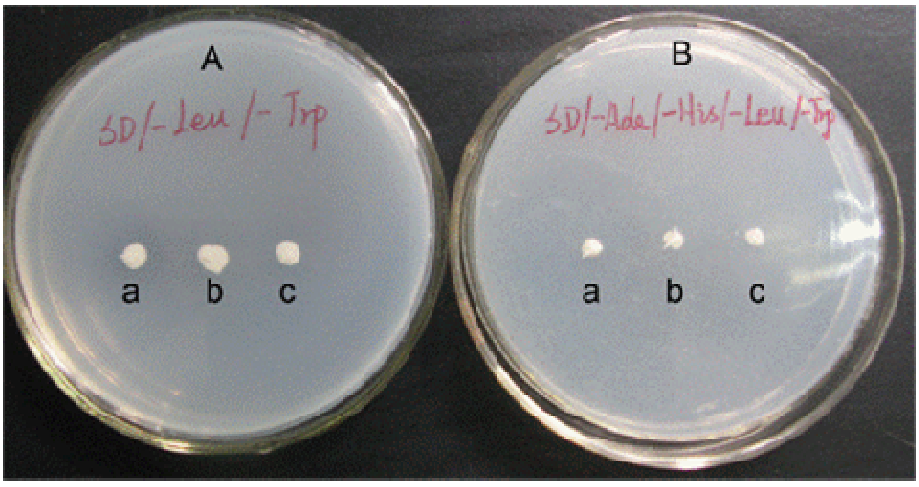

Figure 2. hPer1-Lamr1 interacting detection on SD selection mediums. The interaction between hPer1 and three kinds of Lamr1 were identified with yeast two-hybrid system. Positive clones were grown in SD/–Leu/–Trp (A) and SD/–Ade/–His/–Leu/–Trp (B) selection mediums. Three kinds of clones which contained hPer1 PAS and hLamr11-295 /hLamr11-190/ hLamr1201-295 could grow both in SD/ -Leu/-Trp and SD-Ade/-His/-Leu/-Trp selection mediums. The results revealed that all complete Lamr1 and two partial Lamr1 could interact with hPer1. a: hPer1+Lamr11-295; b: hPer1+Lamr11-190; c: hPer1+Lamr11-295.

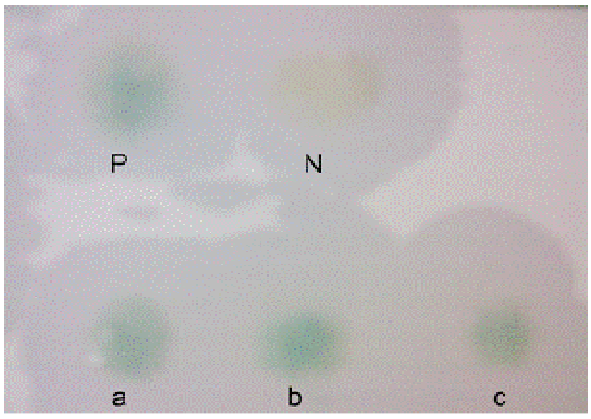

Figure 3. hPer1-Lamr1 interacting detection with colony-lift filter β-galactosidase assay. Colony-lift filter β-galactosidase assay was used to detect the expression of lacZ reporter gene to identify protein-protein interaction. Three kinds of clones which contained hPer1 PAS and hLamr11-295 /hLamr11-190/ hLamr1201-295 performed blue stains in the presence of X-gal, which suggested that all complete Lamr1 and two partial Lamr1 could interact with hPer1. P: Positive Control; N: Negative Control; a: hPer1+Lamr11-295; b: hPer1+Lamr11-190; c: hPer1+Lamr11-295.

hPer1PAS and Lamr1-WML which could grow in both kinds of selection mediums (Figure 5). And the colony-lift filter β-galactosidase assay also revealed that the clones contained hPer1PAS and Lamr1-WML which performed the positive results, while the other two were negative (Figure 6). The results indicated that Lamr1-WML could interact with hPer1, but Lamr1-LL and Lamr1-LMW could not. It suggested that the palindromic sequence LMWWML in peptide G of Lamr1, especially LMW of it, was necessary to the interaction between hPer1 and Lamr1.

DISCUSSION

Circadian rhythms are the overt consequences of biological clocks – endogenous timers acting within cells. At the molecular level, circadian clocks are constructed from ‘clock genes’, some of which encode proteins able to feedback and inhibit their own transcription (Dunlap, 1999). The mammalian clock system is hierarchical, with a master clock located within the neurons of the suprachiasmatic nucleus (SCN) in the hypothalamus. The SCN receives signals from the environment and provides the principal timing cues for synchronizing the daily oscillations in peripheral tissues (Cermakian and Sassone-Corsi, 2000; Panda et al., 2002). The components of the mammalian circadian oscillator involve a complex transcriptional feedback circuit of three period genes (per1, per2 and per3), two cryptochrome genes (cry1 and cry2), a clock gene (clk) and the gene encoding brain–muscle Arnt-like protein 1 (bmal1) (Cermakian and Sassone-Corsi, 2000; Allada et al., 2001; Young and Kay,

2001). As a mammalian ortholog of the drosophila period gene, human period genes (hPer1, hPer2 and hPer3) are considered to be an important component in the mechanism of circadian rhythm (Hida et al., 2000; Shearman et

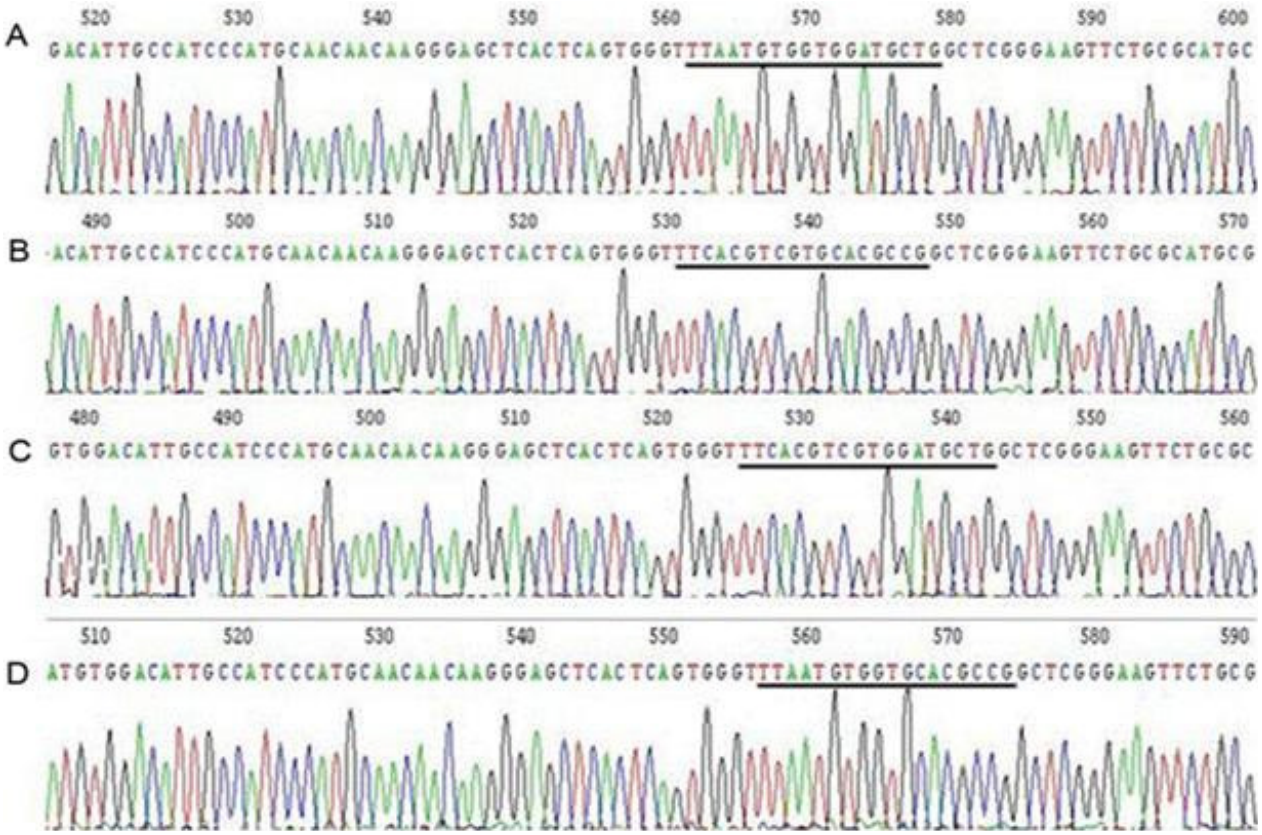

Figure 4. Sequence analysis of mutant plasmids. The DNA sequences of three kinds of mutant plasmids: AD/Lamr1-LL, AD/Lamr1-LMW and AD/Lamr1-WML were analyzed. Sequencing of pGADT7-Rec/hLamr11-190 was performed as control (A). With the mutation, the palindromic sequence LMWWML of hLamr1 was observed as FTSCTP in AD/Lamr1-LL (B), LMW as FTS in AD/Lamr1-LMW (C), and WML as CTP in AD/Lamr1-WML (D). The bars showed the aimed sequences. All the mutate sites were just the mutation we needed, and other sequences were identical with the origin.

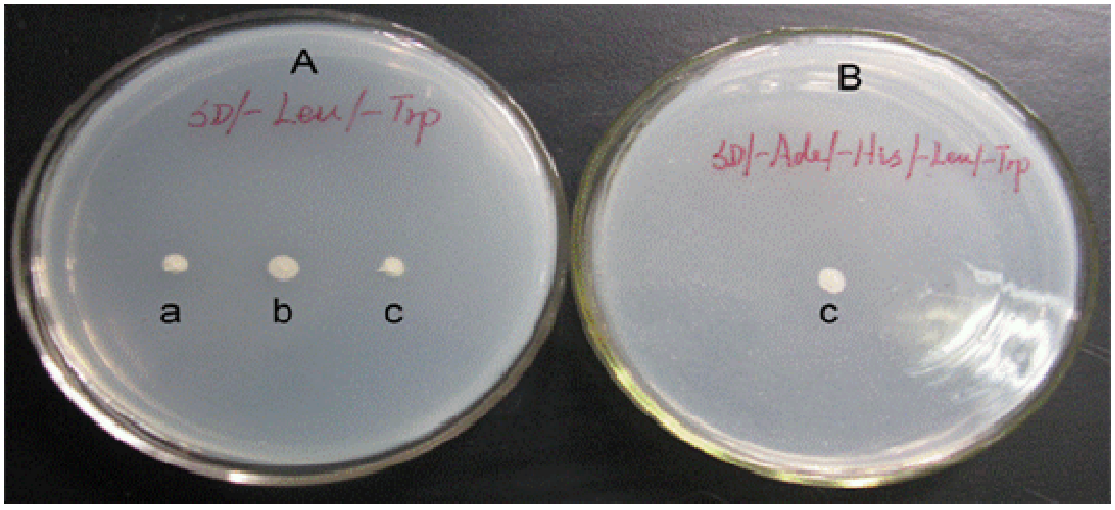

Figure 5. hPer1-mutant Lamr1 interacting detection on SD selection mediums. The interaction between hPer1 and three kinds of mutant Lamr1 were identified with yeast two-hybrid system. Positive clones were grown in SD/–Leu/–Trp (A) and SD/–Ade/–His/–Leu/–Trp (B) selection mediums. The clones that contained hPer1PAS and Lamr1-LL (a) and the clones that contained hPer1 and Lamr1-LMW (b) could grow in SD/ -Leu/-Trp selection mediums, but not in SD-Ade/-His/-Leu/-Trp mediums. However, the clones containing hPer1PAS and Lamr1-WML (c) could grow in both

two kinds of selection mediums. The results indicated that Lamr1-WML could interact with hPer1, but Lamr1-LL and Lamr1-LMW could not. a: hPer1+ Lamr1-LL; b: hPer1+ Lamr1-LMW; c: hPer1+ Lamr1-WML.

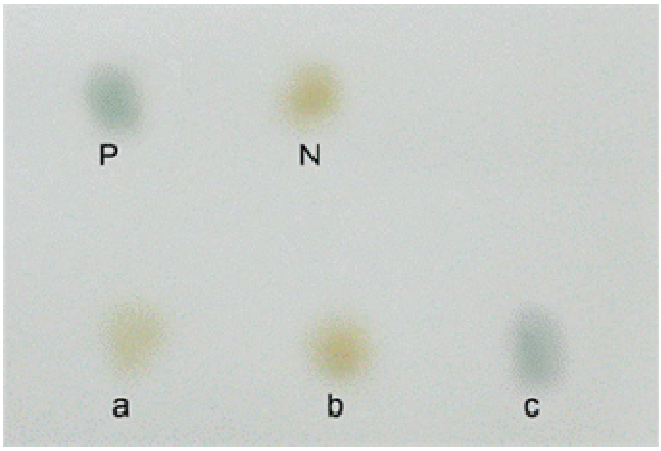

Figure 6. hPer1-mutant Lamr1 interacting detection with colony-lift filter β-galactosidase assay. The clones that contained hPer1PAS and Lamr1-WML (c) performed blue stains in the presence of X-gal, whereas the clones that contained hPer1PAS and Lamr1-LL (a) and the clones containing hPer1 and Lamr1-LMW (b) did not. It revealed that Lamr1-WML could interact with hPer1, but Lamr1-LL and Lamr1-LMW could not. P: Positive Control; N: Negative Control; a: hPer1+ Lamr1-LL; b: hPer1+ Lamr1-LMW; c: hPer1+ Lamr1-WML.

al., 1997). Besides circadian function, Per1 seems to involve many other functions, such as drug dependence and tumor development (Andretic et al., 1999; Liu et al., 2005; Filipski et al., 2002). Because of its complex functions, Per1 may interact with more other proteins to involve intricate functions.

Previous study by Wang et al. finds that hPer1PAS can interact with Lamr1 through yeast two-hybrid system and co-immunoprecipitation analysis, and Lamr1 transcription cannot be influenced by hPer1 (Wang et al., 2007). Lamr1 is a nonintegrin cell surface receptor that mediates high-affinity interactions between cells and laminin. It is found to be widely expressed in most brain, spinal cord neurons, lung, heart, liver, skeletal muscles, thymus, spleen, kidney, intestine and aorta, and to be particularly abundant on the cancer cell surface (Asano et al., 2004; Sobel, 1993). The 67 kDa receptor subunit and its precursor 37LRP appear to play an important role in several physiologic as well as pathologic processes, including cell differentiation, growth, migration and cancer invasion (Faury, 1998; Berno et al., 2005; Qiu et al., 2008; Orihuela et al., 2009). Previous studies show that peptide G (amino acid residues 161-180 of Lamr1) had a high affinity for laminin and Lamr1 binds to laminin, at least in part, via an amino acid sequence contained within that peptide (Castronovo et al., 1991; Magnifico et al., 1996; Taraboletti et al., 1993). And the evolutionary analysis of the sequence identified as the laminin-binding site in the human protein suggests that the acquisition of the laminin-binding capability is linked to the palindromic sequence LMWWML (Ardini et al., 1998). Another binding site is at the carboxyl terminal (amino acids 205–229), which binds to the peptide YIGSR on β1 chain of laminin (Gloe et al., 1999; Landowski et al., 1995). Yeast two-hybrid system is a typical method to identify novel protein-protein interactions, confirm suspected interactions, and define interacting domains *in vivo*. The interactive sequence of Lamr1 to hPer1 is initially identi-fied with yeast two-hybrid system by Wang et al. (2007). They find that the sequence was encoded amino acids from 56 to 295 of Homo sapiens Lamr1 including the sequence which was shown to bind laminin. In the present study, we confirmed the interaction between hPer1 and complete Lamr1 through yeast two-hybrid system. And they found that hPer1 could interact with two partial Lamr1, in which one contained peptide G and the other contained another laminin-binding region. It demonstrated that both two partial sequences contained the interaction region of Lamr1 with hPer1. However, the actual binding sites are not clearly identified.

To further define the key region of Lamr1 to interact with hPer1, we investigated the interaction between hPer1 and Lamr11 -190 mutated with the palindromic sequence LMWWML. The results revealed that hPer1 could not interact with partial Lamr1 mutated with LMWWML, which suggests that the palindromic sequence LMWWML was the binding region of Lamr1 to interact with hPer1. Then we found that hPer1 also could not interact with Lamr1 mutated with partial sequence LMW, but could interact with Lamr1 mutated with WML, suggesting that the amino acid sequence LMW of Lamr1 was necessary to the interaction. While, the palindromic sequence LMWWML is just the actual binding site for laminin in peptide G of Lamr1. Together, these findings suggested that hPer1 might interact with Lamr1 by occupying the laminin-binding sites. It will be beneficial for studying the mechanism of hPer1 interaction with Lamr1. However, we only proved one binding domain; more regions need to be proved to confirm the conclusion.

With yeast two-hybrid system and site-directed mutagenesis, we found that the palindromic sequence LMWWML of Lamr1, especially LMW of it, was necessary to the interaction of Lamr1 and hPer1. The finding suggested that hPer1 might interact with Lamr1 by occupying the laminin-binding sites. It will provide a new way to study the complex function of hPer1 and Lamr1. For example, the present study may offer a new theory for tumor development and therapy. But more evidence need to be provided in further studies. And though Lamr1 transcription could not be influenced by hPer1, whether hPer1 could affect the function of Lamr1, or whether Lamr1 could influence hPer1 transcription or its function. These problems need to be solved in future research.

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Full Length Research Paper

Detection of *Trypanosoma brucei gambiense* and *T. b. rhodesiense* in *Glossina fuscipes fuscipes* (*Diptera: Glossinidae*) and *Stomoxys* flies using the polymerase chain reaction (PCR) technique in southern Sudan

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Ethanol-fixed entire bodies of the tsetse fly, *Glossina fuscipes fuscipes*, and unidentified stable flies, *Stomoxys* spp., collected from near Juba town, southern Sudan, were tested for *Trypanozoon* trypanosomes infections using polymerase chain reaction (PCR) technique for the first time in Sudan. The crude target DNA sequences were extracted by incubation of entire flies in Nonindet PCR template buffer containing proteinase-K. The DNA amplification sets of conditions were adjusted for each pair of primers employed. The oligonucleotide primers used included TBR_{1-2}, SRA_{A-E}, $SRA_{B537-B538}$ and $TgsGP_{FOR-REV}$. The results showed that 74.4% of *G. f. fuscipes* and 39.36% of *Stomoxys* spp. were infected with *Trypanozoon* trypanosomes. Out of the 117 examined *G. f. fuscipes*, 46.2, 24.8, 35.04, 17.09 and 10.26% were due to *T. b. gambiense* ($TgsGP_{FOR-REV}$), *T. b. rhodesiense* (SRA_{A-E}), *T. b. rhodesiense* ($SRA_{3537-3538}$), mixed infection with *T. b. gambiense* and *T. b. rhodesiense* and *T. b. brucei*, respectively. However, infections in *Stomoxys* spp. of 2.13 and 37.2% were due to *T. b. rhodesiense* and *T. b. brucei*, respectively.

Key words: *Glossina fuscipes fuscipes*, *T. b. gambiense*, *T. b. Rhodesiense*, vectoral capacity, infection rate, PCR technology.

INTRODUCTION

At the end of the 19th century, sleeping sickness or human african trypanosomosis (HAT) decimated about a quarter million people in central Africa including the whole of Uganda, Kenya shores and islands of Lake Victoria, Rwanda/Burundi, the Congo and northward into Equatoria Province of southern Sudan. This devastating epidemic was attributed to *Trypanosoma brucei gambiense* or the Gambian form of the disease (Bloss, 1960; Ford and Katondo 1971). However, at the time of the epidemic there were no alternative effective diagnostic tools, which might have led to the suspicion that the causative agent was anything other than *T. b. gambiense*. Nevertheless an outbreak of epidemic proportions attributed to *T. b. rhodesiense* was reported in sudanese/Ethiopian border in the early 1970s (Baker *et al*., 1974). Considering the current instability of people and livestock due to the latest war, there is a high probability of the spread and overlap of the two types of sleeping sickness in the rest of southern Sudan.

Although seven species of *Glossina* were recognized in Sudan (Lewis, 1949), *Glossina f. fuscipes* has been incriminated as the only vector of *T. b. gambiense*, the causative agent of HAT in southern Sudan (Snow, 1983). Empirical data have also shown that some biting flies can transmit pathogenic trypanosomes, but only few species in nature are considered epidemiologically important (Leak and Rowlands, 1997). Obtaining accurate knowledge of the dynamics of natural trypanosome infections in vector

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Abbreviations: PCR, Polymerase chain reaction; **HAT,** human African trypanosomosis.

Table 1. The PCR reaction buffer compositions.

Materials (1rxn)	TBR_{1-2} (µl)	SRA (µl)	$TgsGP_{FOR-REV}$ (µl)
10x buffer	2	2	2
200µM for each dNTPs	2	2	0.5
1 µM specific primer -1	0.5	0.5	0.5
1 µM specific primer - 2	0.5	0.5	0.5
2.5 unit *Taq* DNA polymerase	0.3	0.2	0.5
Crude template DNA	2	2	2
Sub-total volume	7.3	7.2	6
De- ionized water	12.7	12.8	14

Insects and the adventitious host will significantly contribute to a better understanding of the epidemiology of trypanoso-moses (Woolhouse et al., 1993).

The classical techniques used to detect trypanosome infection in flies (Lloyd and Johnson, 1924) are of limited sensitivity and specificity (Jordan, 1974; Woolhouse et al., 1994). Currently, trypanosome-detection techniques have significantly improved by using very high specific molecular tools such as the Polymerase chain reaction (PCR) (Majiwa and Otieno, 1990; Masiga et al., 1992; McNamara et al., 1995). In this paper we present results of experiments performed to identify and estimate the natural *Trypanozoon* trypanosomes infection rate of *Glossina fuscipes fuscipes* and *Stomoxys* spp in Juba area, southern Sudan using PCR technique (Masiga et al., 1992). The area had an active focus of sleeping sickness during the time of the study.

MATERIALS AND METHODS

Study area

The flies were collected from locations, near Juba town (4°40` - 5°N; 30°30'- 3°45`E), Central Equatoria, Southern Sudan. The area has an equatorial or semi-equatorial climate. The most dominant feature in the area is River Bahr El Jebel, which originates from Lake Victoria. Patches of evergreen thickets and double storey gallery forests grow along the banks of the river, and are infested with *Glossina fuscipes fuscipes*. The main trees of the gallery forest are *Ficus religiose, Tamarindus indica, Azadirachta indica, Anogeissus leiocarpus* together with *Combretum* spp. and various climbers and grasses. This riverine vegetation is however interrupted by several villages, hamlets and small numerous plots for subsistence farming. In most cases these plots have thick green hedges, which are deemed suitable habitats for *G. f. fuscipes* together with other biting flies including *Stomoxys* spp.

Capture of flies

G.f. fuscipes and *Stomoxys spp.* were caught in five unbaited biconical traps (Challier et al., 1977). Traps were placed 200 m apart and catches were collected at 24 h intervals for three consecutive days. Captured flies were killed, counted and examined for tenerality. Only non-teneral male and female tsetse flies and 10% of engorged male and female *Stomoxys spp.* were used. These were preserved separately in ethanol in an eppendorf tube until examined for trypanosome infection using PCR technique (EANETT, 2005).

Preparation of crude DNA templates

The ethanol was suck using a sterilized pipette. The fixed flies were left to dry by air. Then each fly was crushed under liquate nitrogen using a sterilized mortar and incubated in 100 µl template preparation buffer (10 mM Tris-Cl pH 8.3; 50 mM KCl; 1.5 mM MgCl; 0.9% Nonindet P40). 25 µl Proteinase - K was added to each tube at concentration of 60 mM. Thereafter, the mixture was incubated at 55°C for one hour in a water bath. The proteinase was denatured by increasing the temperature to 95°C for 10 min. Such preparations were either used immediately or stored at -20°C.

Primers

The oligonucleotide primers used (Table 1) to amplify the target DNA sequences includes TBR_{1-2} (Moser et al., 1989), SRA_{A-E} (Gibson et al., 2002), $SRA_{B537-B538}$ (Welbum et al., 2001) and $TgsGP_{FOR-REV}$ (Radwanska et al., 2002).

PCR cycling

PCR amplifications were carried out in 20 µl reaction mixture. The reaction buffer compositions were adjusted for each pair of primers used as shown in Table 1. The reaction mixtures were cycled in a programmed PCR machine for each pair of primer used as follows: for TBR-primers; initial denaturation 94°C/3 min (1 cycle), denaturation 92°C/30 s, annealing 60°C/45 s, extension 72°C/45 s (30 cycle), final extension 72°C/5 min (1 cycle). For SRA-primers; initial denaturation 95°C/3 min (1 cycle), denaturation 95°C/30 s, annealing 60°C/30 s, extension 72°C/60 s (35 cycle), final extension 72°C/5 min (1 cycle). For TgsGP-primers; initial denaturation 95°C/5 min (1 cycle), denaturation 94°C/45 s, annealing 63°C/45s, extension 72°C/120 s (45 cycle), final extension 63°C/10 min (1 cycle). Then 20 µl of each sample was electrophoresed through 1.5 - 2% agarose containing 0.5 µg ml^{-1} Ethidium-bromide and the voltage was set at 60 V for the electrophoretic mobility to visualize the amplified DNA and compared to a standard DNA consisting of a known amount of bp.

RESULTS

Overall the biconical traps caught 249 and 938 *G. f. fuscipes* and *Stomoxys* spp., respectively. A number of

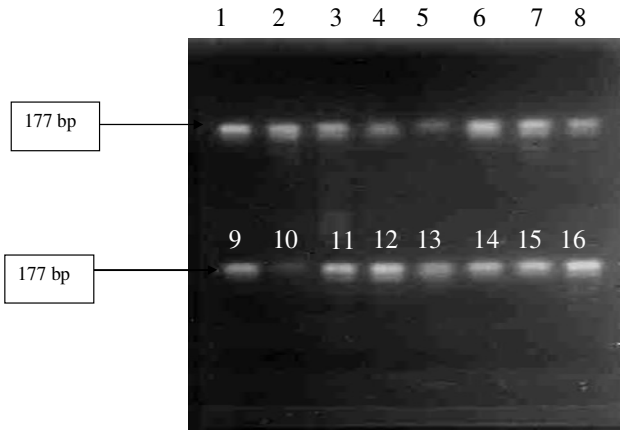

Figure 1. Ethidium-bromide stained 1.5% agarose gel showing PCR amplification of crude template of whole *Glossina fuscipes fuscipes* and *Stomoxys* flies using TBR_{1-2} primer sets: 1-16 *Trypanosoma brucei* lanes show 177 bp using ΦX174 Hae III Digest 100-bp marker.

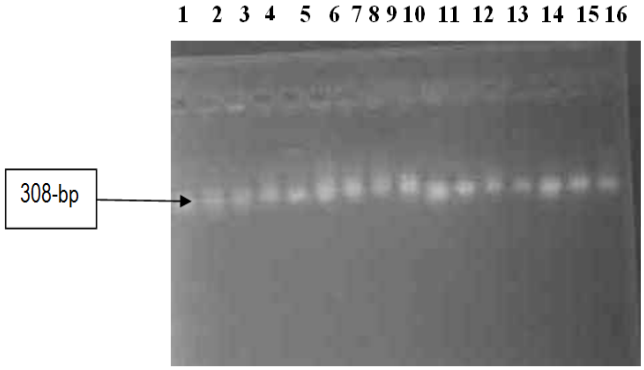

Figure 2. Ethidium-bromide stained 1.5% agarose gel showing PCR amplification of crude template of *Glossina fuscipes fuscipes* females using $TgsGP_{FOR-REV}$. 1-16 *T.b.gambiense* lanes show 308-bp using ΦX174 Hae III Digest 100-bp marker.

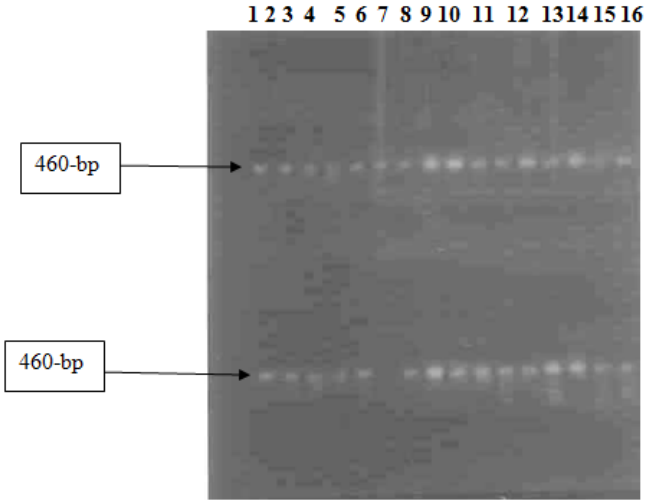

Figure 3. Ethidium-bromide- stained 1.5% agarose gel showing PCR amplification of crude template of *Glossina fuscipes fuscipes* males using SRA_{A-E} and $SRA_{3537-3538}$ pair primer sets: 1-16 first row *T. b. rhodesiense* SRA_{A-E} and1-16 second row *T. b. rhodesiense* $SRA_{3537-3538}$ lanes show 460-bp using ΦX174 Hae III Digest 100-bp marker.

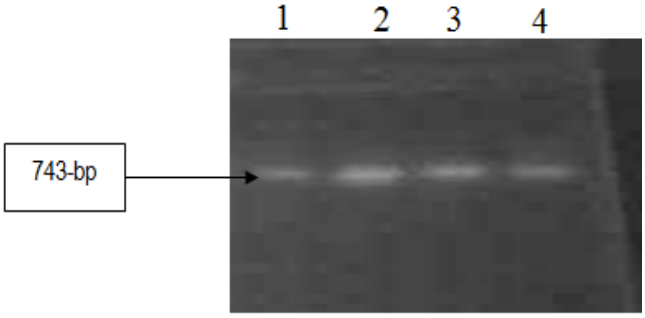

Figure 4. Ethidium-bromide - stained 1.5% agarose gel showing PCR amplification of crude template of *Stomoxys* using $SRA_{B537-B538}$ primer sets. 1,2,3,4 replicates *T. b. rhodesiense* lanes show 743-bp using ΦX174 Hae III Digest 100-bp marker

117 non-teneral male and female *G. f. fuscipes* and 94 engorged male and female *Stomoxys* spp were examined using $TBR_{1-2,}$ SRA_{A-E}, $SRA_{B537-B538}$ and $TgsGP_{FOR-REV}$ $SRA_{B537-B538}$ primers for infection with *Trypanozoon* trypanosomes. Among the 117 *G. f. fuscipes* examined, 87 reacted positively with TBR_{1-2} primers: 54, 29 and 41 reacted positively with $TgsGP_{FOR-REV}$, SRA_{A-E} and $SRA_{B537-B538}$ pair primers, respectively, while 20 of the samples reacted positively with the three set of primers used. In contrast 37 and 2 out of 94 *Stomoxys* flies reacted positively with $TBR_{1-2,}$ and primers, respectively. Amplification products of expected band size for *Trypansoma brucei* group, *T. b. gambiense*, and *T. b. rhodesiense* are clearly visible in Figures 1, 2, 3, and 4.

The analysis of the results obtained (Tables 2 and 3) indicated that the overall infection rate of *Trypanozoon* was 74.4% based on the number of the non-teneral *G. f. fuscipes* flies. The burden of flies harboring *T. b. gambiense* trypanosome was 46.2% (54/117). Of the 54 infected flies, 34 were infected with *T. b. gambiense* and 20 with *T. b.* $SRA_{B537-B538}$ *gambiense* and *T. b. rhodesiense*. The rate of flies infected with *T. b. rhodesiense* using SRA_{A-E} and primers was 24.8% (29/117) and 35.04% (41/117), respectively: Of the 29 flies that reacted with SRA_{A-E}, 20 flies harboring *T. b. gambiense* and *T. b. rhodesiense* mixed infection; Of the 41 flies that reacted with $SRA_{B537-B538}$, 21 flies reacted as well with SRA_{A-E} and 20 flies harboring *T. b. gambiense* and *T. b. rhodesiense* mixed infection.

The overall mixed infection of *T. b. gambiense* and *T. b. rhodesiense* was 17.09% (20/117). The remaining

Table 2. *Trypanozoon* infection rates detected by PCR technique in wild *G. f. fuscipes* and *Stomoxys* flies.

Fly spp.	TBR_{1-2}	$TgsGP_{FOR-REV}$	SRA_{A-E}	$SRA_{B537-B538}$	Multiple
G.f.fuscipes	74.4%(87/117)	46.2%(54/117)	24.8%(29/117)	35.0%(41/117)	17.0%(20/117)
stomoxys	39.4%(37/94)	0	0	2.13%(2/94)	0

Table 3. The number of wild *G. f. fuscipes* and *Stomoxys* flies infected with *Trypanozoon* trypanosomes detected by PCR technique.

	TBR_{1-2}	$TgsGP_{FOR-REV}$	SRA_{A-E}	$SRA_{B537-B538}$
TBR_{1-2}	12	54	29	41
$TgsGP_{FOR-REV}$	54	34	20	20
SRA_{A-E}	29	20	9	29
$SRA_{B537-B538}$	41	20	29	12

infection was attributed to *T. b. brucei* of 10.26% (12/117). None of the *Stomoxys* flies were found infected with *T. b. gambiense*, nevertheless, 2.13% (2/94) and 37.2% (35/94) of these *Stomoxyinae* were infected with *T. b. rhodesiense* and *T. b. brucei*, respectively.

DISCUSSION

The present work shows that *G. f. fuscipes* is the potential vector of sleeping sickness ascribed to *T. b. gambiense* or *T. b. rhodesiense* in the study area and *Stomoxys* species may play a role in transmission of *T. b. rhodesiense* because they usually feed on animals as well as humans. The *G. f. fuscipes* is probably the only vector of sleeping sickness; this is because no other *Glossina* spp. had been found in the area. According to Ford (1963) and Ford and Katondo (1977), the distribution of *G. f. fuscipes* overlaps with that of *G. morsitans submorsitans* in the study area in southern Sudan. However, recently only *G. f. fuscipes* was detected in the area (Mohammed, 2004). Although the lack of capture of *G. m. submorsitans* could as well be due to the absence of this species in the area, the use of the bi-conical trap as the only catching device and without odour bait might have also contributed to the dearth in catches *G. m. submorsitans* (Mohamed-Ahmed et al., 1993).

The species-specific oligonucleotide primers for PCR amplification becomes obtainable for different trypanosomes. Consequently, correct identification and classification of trypanosomes in archived samples, tsetse and other biting flies is possible (Stijn et al., 2008). In view of the fact that the amplification of the crude preparations of templates from a whole fly produced clearly visibly an expected band size for *Trypansoma brucei* group, *T. b. gambiense*, and *T. b. rhodesiense* is an indication of infection. Thus, this indicates that tsetse flies of *G. f. fuscipes* are the only vectors transmitting human african Trypanosomosis in the study area. To date no evidence has been reported elsewhere in Africa of biological transmission of trypanosomes responsible for HAT in vectors other than *Glossina* (Leak, 1999). Conversely, there was ample evidence that biting flies *Stomoxys* are responsible for the maintenance and spread of Nagana diseases causative trypanosomes including *Trypanozoon* (Karib, 1961; Abdulla et al., 2005).

The PCR technique (Masiga *et al*., 1992; EANETT, 2005) was used in the present study to identify and detect trypanosomes infections in tsetse and stable flies. This was done in order to estimate the role of *G. f. fuscipes* and *Stomoxys* spp. in the epidemiology of HAT in southern Sudan as the traditional trypanosome detection techniques (Lloyd and Johnson, 1924; Mohamed-Ahmed *et al*., 1989) which rely on microscopic examination could identify parasites to the subgenus level only and would not differentiate between *brucei rhodesiense, brucei gambiense* or *brucei brucei* trypanosomes.

Some authorities base infection rates on the number infected out of the total non-teneral male and female flies whereas others base infection rates on total flies examined including tenerals (Jordan, 1974; Mihok et al., 1992). In the present work infection rates was based on non-teneral *G. f. fuscipes* flies examined for infection using PCR. This was done because non-teneral tsetse flies are more likely to get infected than teneral ones which have yet to obtain their first blood meal (Welburn and Maudlin, 1992).

Recent and previous devastating sleeping sickness epidemics in sudan were attributed to the Gambian form of the disease only (Bloss, 1960) although at the time of those epidemics, effective diagnostic techniques such as the PCR were not developed to verify the exact cause. Moreover, Sudan lies in the interface of the geographical belt of both types of diseases and that *T. b. rhodesiense* had caused an epidemic outbreak in Sudan eastern border (Baker et al., 1974).

The present results indicate that *G. f. fuscipes* and *Stomoxys* spp found in Bahr El Jebel State were infected

with *T. b. rhodesiense*. This suggests that the *rhodesiense* form of the disease might have been present in the area but had been overlooked due to inefficient diagnostic techniques in both human and vectors.

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Full Length Research Paper

Biosorption of mercury by capsulated and slime layer-forming Gram -ve bacilli from an aqueous solution

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The biosorption of mercury by two locally isolated Gram-ve bacilli: *Klebsiella pneumoniae* ssp. *pneumonia* (capsulated) and slime layer forming *Pseudomonas aeruginosa*, was characterized. Mercury adsorption was found to be influenced by the pH value of the biosorption solution, initial metal concentration, amount of the dried biomass and contact time. The optimum biosorption capacity of *K. pneumonia*e (about 15%) was recorded at pH 5, initial mercury concentration of 0.1 g/L and when contacted for less than 60 min with 1.0 g dried cells/L. While, the highest biosorption capacity of *P. aeruginosa* (about 25%) was reached at pH 5.8, initial mercury level of 0.15 g/L and for less than 60 min contacted with 1.0 g dried biomass/L. The efficiency average of slime layer forming *P. aeruginosa*, of high negatively charged components, showed more than 1.5 fold increase as compared to capsulated *K. pneumonia*e of low negatively charged constituents, under all the tested characteristics of mercury biosorption from aqueous solution.

Key words: Biosorption, mercury, *Klebsiella pneumoniae, Pseudomonas aeruginosa,* capsulated and slime forming bacilli.

INTRODUCTION

Mercury is one of the most toxic heavy metals released in the environment (Shaolin and David, 1997; Zilloux et al., 1993). The major effects of mercury poisoning are neurological and renal disturbances, as well as impairment of pulmonary function (Manohar et al., 2002; Saglam et al., 2002). This metal is released to the environment from anthropogenic activities that include agriculture, battery production, fossil fuel burning, mining and metallurgical processes, paint and chloralkali industries, and wood pulping (Boening 2000). In addition, activities such as solid waste combustion, industrial developing and petrochemical activities discharge mercury to the environment, especially aquatic ecosystem (WHO, 1998). Finding an effective method of removal of toxic heavy metals from industrial waste water is essential from the stand point of environmental pollution control (Green-Ruiz, 2006; Khoramabadi et al., 2008; Say et al., 2003; Svecova et al., 2006).

Conventional techniques for the removal of heavy metals from wastewater, such as chemical precipitation, ion exchange, activated carbon adsorption and separation processes have limitations and become inefficient and expensive especially when the heavy metal concentration is less than 100 ppm (Yan and Viraraghavan, 2001). The use of inexpensive biological materials, such as microorganisms, for removing and accumulating heavy metals from contaminated aqueous solutions has been widely reported (AL-Garni et al., 2009; EL-Sherif et al., 2008; Green-Ruiz, 2006; Khoramabadi et al., 2008; Lopez-Errasquin and Vazquez, 2003; Svecova et al., 2006; Zamil et al., 2009). The uptake of metals by biomass can take place actively, by means of metabolic activity-dependent process (bioaccumulation) or by means of a passive and usually rapid (several minutes) metabolism-independent process called biosorption (Godlewska-Zykiewicz and Kozowska, 2005). Inactivated, nonliving microbial biomass seems to be more advantageous than using living cells (Al-Garni et al., 2009; Gadd, 1992) and can serve as a basis for the development of potent biosorbent materials of strategic or valuable heavy metals

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and has a potential application in environmental control of these toxicants (Volesky, 1990). The mechanism and kinetics of metal biosorption on biomass depends on the experimental conditions particularly, medium pH, initial metal ion concentration, biomass concentration and affinity of the binding sites for the metal (Converti et al., 2006; Zeroual et al., 2003).

Klebsiella pneumoniae is a Gram -ve bacillus that possesses a thick polysaccharide capsule of glucose, mannose, galactose, glucuronic and galacturonic acids and lacks phosphoryl residues (Fresno et al., 2006; Kobayashi et al., 2002). While, *Pseudomonas aeruginosa* is also a Gram -ve bacillus that possesses a slime polysaccharide layer of rhamnose, glucose, mannose, glucosamine, galactsamine, glucuronic acid and acetyl groups (Arsenis and Dimitracopoulos, 1986; Bartell et al., 1970). *K. pneumoniae* was successively used for the biosorption of lead (Al-Garni, 2005) and *P. aeruginosa* was studied for the biosorption of mercury (Hassen et al., 1998). The objectives of the present work are: first, is to characterize some physicochemical parameters of mercury biosorption by local isolates of *K. pneumoniae* ssp. *pneumonia* and *P. aeruginosa*, and second, to evaluate the efficiency of capsule and slime layer forming Gram -ve bacilli for mercury biosorption from polluted liquids.

MATERIALS AND METHODS

Microorganisms and maintenance

K. pneumoniae ssp. *pneumonia* and *P. aeruginosa*, local isolates, were kindly provided by Microbiology Laboratory, king Abdul-Aziz Medical City, Jeddah, Saudi Arabia, and their identification was routinely assessed using PHONIX 100BD and MICRO SCAN walk away 9651 apparatus.

Growth and preparation of the dried powdered dead cells

The tested bacteria were maintained on nutrient agar slants. The stock cultures were transferred weekly and stored at 10°C. Biomass of the tested bacteria was developed by growing in MacConky broth medium (Nentech, LTD, U.K.), as a selective medium for the tested genera, at pH 7.0, incubated for 48 h at 37 ± 1°C, under shaken conditions (120 rpm). Cells were harvested by centrifugation at 8000 rpm for 10 min (Sorvall Ultra Pro 80, Surespin 630, Kendro Laboratory, USA). Harvested cells (biomass) were washed twice with deionized distilled water and dried in an oven at 80°C for 48 h (Puranik and Pakniker, 1999). To assess complete death of the dried cells, samples of the dried cells were inoculated to Petri-dishes containing MacConky agar medium, absence of any growth indicated positive results (complete death of the bacteria). The dried cells were then grinded in a blinder and sieved to obtain a fine powder (0.2 mm), and stored at 5°C until use.

Metal solution

A stock mercury ion solution (1000 mg/L) was prepared by dissolving mercuric chloride ($HgCl_2$) (Fisher Scientific Ltd) in deionized distilled water (Chen et al., 2005). Solutions were adjusted to the desired pH values using 0.1 N NaOH and 0.1 N HCl (Svecova et al., 2006). The mercury concentration was determined using atomic absorption spectrophotometer (Unicam 929AA).

Metal absorption studies

A batch equilibrium method was used to determine sorption of mercury by the tested bacteria. All biosorption experiments were conducted in 250 mL Erlenmeyer flasks containing 100 mL of the tested mercury solution. Powdered biomass (100 mg, unless otherwise stated) was exposed to metal solution for 60 min (otherwise stated) at 25 ± 2°C on a rotary shaker at 120 rpm. The biomass was separated by centrifugation at 10000 rpm for 10 min, and residual mercury concentration was measured in the supernatant. Metal adsorbed by the biomass (mg metal/g biomass) was calculated (Volesky and May-Phillips 1995) as:

$$Q = V (Ci – Cf) / 1000 \times M$$

Where, Q = mercury uptake (mg Hg (II)/mg biomass), V = volume of mercury solution (mL), Ci = initial Hg (II) concentration (mg/L), Cf = final Hg concentration (mg/L) and M = mass of powdered dried cells (mg).

Effect of pH

To test the effect of pH value on Hg (II) biosorption, the dried powdered cells of *K. pneumoniae* ssp. *pneumonia* and *P. aeruginosa* were suspended in mercury solution with pH values ranging from 2 - 7 for 60 min on a rotary shaker at 120 rpm. Thereby, the necessary analyses were carried out.

Effect of initial mercury concentration

Metal solutions (100 mL) of varying concentrations of mercury (25 - 400 mg/L) adjusted to the optimum pH of 5 for *K. pneumoniae* ssp. *pneumonia* and pH 5.8 for *P. aeruginosa*, were treated with 100 mg of the dried powdered cells of the test bacteria. Thereby, the biosorption was completed and necessary analyses were carried out.

Effect of dried powdered cells concentration

Dried powdered cells (100 – 1000 mg) were exposed to 100 mL of mercury solution (100 mg/L) at the optimum pH 5 for *K. pneumoniae* ssp. *pneumonia* and (150 mg/L) at pH 5.8 for *P. aeruginosa* for 60 min on a rotary shaker at 120 rpm. Thereby, the residual mercury in the supernatant after centrifugation at 10000 rpm for 10 min was measured using atomic absorption spectrophotometer.

Effect of contact time

To examine the mercury biosorption mechanism, 100 mg of dried powdered cells of the bacteria were contacted with 100 mL aliquots of mercury solutions (100 mg/L for *K. pneumoniae* ssp. *pneumonia* and 150 mg/L for *P. aeruginosa*) in 250 ml Erlenmeyer flasks. The flasks were incubated at 25 ± 2°C for different time intervals (5 – 180 min), thereafter analyzed for residual mercury content.

All experiments were carried out in triplicates and the recorded results are the arithmetic mean.

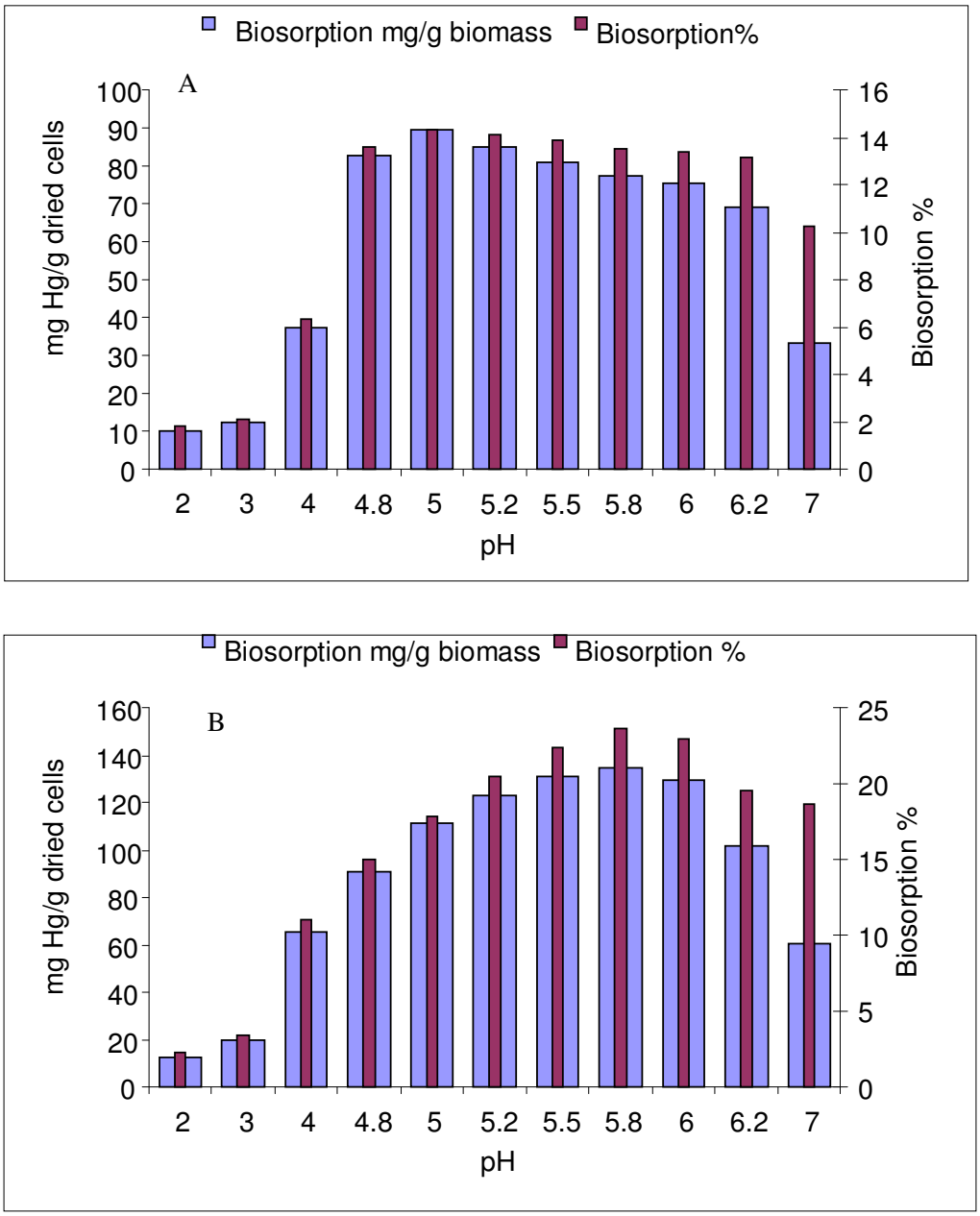

Figure 1. Mercury biosorption (mg Hg/g dry cells and as %) by dry biomass of *K. pneumoniae* (A) and *P. aeruginosa* (B) as influenced by the pH value.

RESULTS AND DISCUSSION

Effect of pH values on Hg (II) biosorption

The results (Figure 1) revealed that a pH range of 4.8 to 5.2, (especially pH 5) was optimum for biosorption of Hg by *K. pneumoniae*, where maximum Hg biosorption (14.36%) was recorded at pH 5. However, a pH range of 5.5 to 6 was optimum for Hg biosorption by *P. aeruginosa* and pH value of 5.8 proved to be the most suitable for maximum Hg sorption (23.61%) by the bacterium. The results indicated that *P. aeruginosa* was more efficient to biosorb Hg, 64.4% more than *K. pneumoniae*. It was reported that pH value has an important role in metal ions biosorption (Al-Garni et al., 2009; Bae et al., 2002; Green-Ruiz, 2006), where the active biosorbing groups have the ability to accept or loss protons that depend mainly on the pH value (Yalcinkaya et al., 2002). Also, it affects metal ions solubility and ionization of the biosorbing groups in the biomass surfaces (Fourest and Volesky, 1996). It was also reported that high acidity makes the biosorbent surface accept protons (H^+) and

therefore reduce their ability to adsorb positive Hg ions and inversely, decrease acidity to its optimum value, which differ from one biomass to another and type of adsorbed metal ions. The adsorbing surface saturated with negative charges, resulted in increased efficiency to bind and adsorb metal ions of positive charges (Bayramoglu et al., 2003). The pH affects the network of negative charges on the surface of the biosorbing cells and the chemistry of the walls, as well as physicochemistry and hydrolysis of the metal (Collins and Stotzky, 1996; Lopez et al., 2000).

In accordance with our finding that Hg adsorption was low at low and high pH values by the tested bacteria, it was reported that at low pH values, adsorption of metals decreases because of competition for binding sites between cations (as Hg^{++}) and protons (Sahoo et al., 1992), while at pH higher than 7, hydroxo species of the metals can be formed and do not bind to the adsorption sites on the surface of the adsorbent (Kacar et al., 2000). The higher efficiency of *P. aeruginosa* to absorb Hg than *K. pneumoniae* at the different tested pHs may be due to the high content of reducing sugars, amino sugars, carboxylic acids, deoxysugars and acetyl groups of the slime layer of *P. aeruginosa* (Arsenis and Dimitracopoulos, 1986; Bartell et al., 1970) as compared to the capsule content of *K. pneumoniae* of non-polar lipopolysaccharides, carboxylic acids and amino sugars (Fresno et al., 2006), instead of the two bacteria which are Gram -ve bacilli. It may also be due to the presence of compact dense capsule of *K. pneumoniae* which may hide and/or block some adsorbing surfaces on the bacterial cell wall and make them unavailable to bind to the positive metal ions, as compared to the loose slime layer of *P. aeruginosa*.

Effect of initial mercury concentration on biosorption

The effect of initial concentrations of Hg (II) (25 – 400 mg/L) on its biosorption by nonviable cells of *K. pneumoniae* ssp. *pneumonia* and *P. aeruginosa* was evaluated. The results (Figure 2) indicated that in terms of percentage, the removal of mercury from the solutions appeared to be more efficient at 100 mg Hg/L for *K. pneumoniae* and at 150 mg Hg/L for *P. aeruginosa* (28.65 and 29.83%, respectively) than at lower or higher metal concentrations. The enhancement in metal sorption could be due to an increase in electrostatic interactions, involving sites of progressively lower affinity for metal ions (Al-Asheh and Duvnjak, 1995; Puranik and Pakniker, 1999). These data indicated that mercury uptake by the two tested Gram -ve bacteria (capsulated and slime forming) was chemically equilibrated and saturation was attained at an initial Hg(II) of 100 mg/L for *K. pneumoniae* and at 150 mg Hg/L for *P. aeruginosa*. So, there was no increase in metal uptake where the binding sites were saturated by the metals. The results indicated that the adsorbing sites for mercury of *P. aeruginosa* are more than that of *K. pneumoniae* by more than 50%, whereas, the adsorbing sites of *K. pneumonia*e was saturated by 100 mg Hg(II)/L and with adsorbing percentage of 28.65%, while the adsorbing surfaces of *P. aeruginosa* was equilibrated by 150 mg Hg(II)/L (with 29.83% adsorption). At the same experimental conditions, this may due to the high content of negatively charged compounds of its slime layer, as compared to that of *K. pneumoniae* capsule with less negatively charged cons-tituents.

It was reported by many workers that increasing initial mercury concentrations in liquids resulted in increasing its biosorbing efficiency until saturation of adsorbing sites, followed by lower adsorption efficiency (Green-Ruiz, 2006; Kacar et al., 2000; Saglam et al., 2002).

Effect of dried powdered cells level on biosorption

The results given in Figure 3 shows that the specific metal uptake (mg Hg/g cell mass) at different concentrations (1 to 10 g/L) of dried powdered cells of *K. pneumoniae* and *P. aeruginosa* were decreased with increasing dry mass concentrations. Thus, about 58.5 and 70.7% decreases were recorded for *K. pneumoniae* and *P. aeruginosa*, respectively, as the dry mass increased from 1 to 10 g/L. This could be attributed to interference between binding sites at higher mass levels (De Rome and Gadd, 1987). Reduction in metal uptake by the biosorbent with increasing biomass concentration was also attributed to an insufficiency of metal ions in solution with respect to available binding sites (Al-Asheh and Duvnjak, 1995; Fourest and Roux, 1992; Sampedro et al., 1995). Higher specific uptake at lower dry mass concentrations could be due to an increased metal-to-biosorbent ratio, which decreases upon an increase in dry mass concentration (Puranik and Pakniker, 1999). The obtained results indicated that the number of adsorbing negative charges on the biomasses' levels (1 g/L) of both bacteria was at equilibrium with metal ions, therefore increase in biomass level is not accompanied by increasing adsorption efficiency (Al-Garni, 2005; Al-Garni et al., 2009; Mashitah et al., 2008; Tawfik et al., 2005). However, as the biosorbed Hg was calculated on the basis of percentages, as expected, it was increased regularly with increasing dry mass of the cells. Thus, as the mass increased from 1 to 10 g/L about 4.2 and 2.9 fold increases in the percentage of biosorbed Hg (II) were recorded with *K. pneumoniae* and *P. aeruginosa*, respectively. However, in the basis of specific Hg uptake (mg Hg/g cell mass) about 2.4 and 3.4 fold decrease were recorded, respectively. This is in accordance with previous work in which it was reported that increased biomass concentration of the microbial cells was attained with increased metal sorption as g/L, and in the basis of absorbed metal as mg/g cell mass biosorption decreased regularly (Al-Garni, 2005; Gupta and Keegan, 1998;

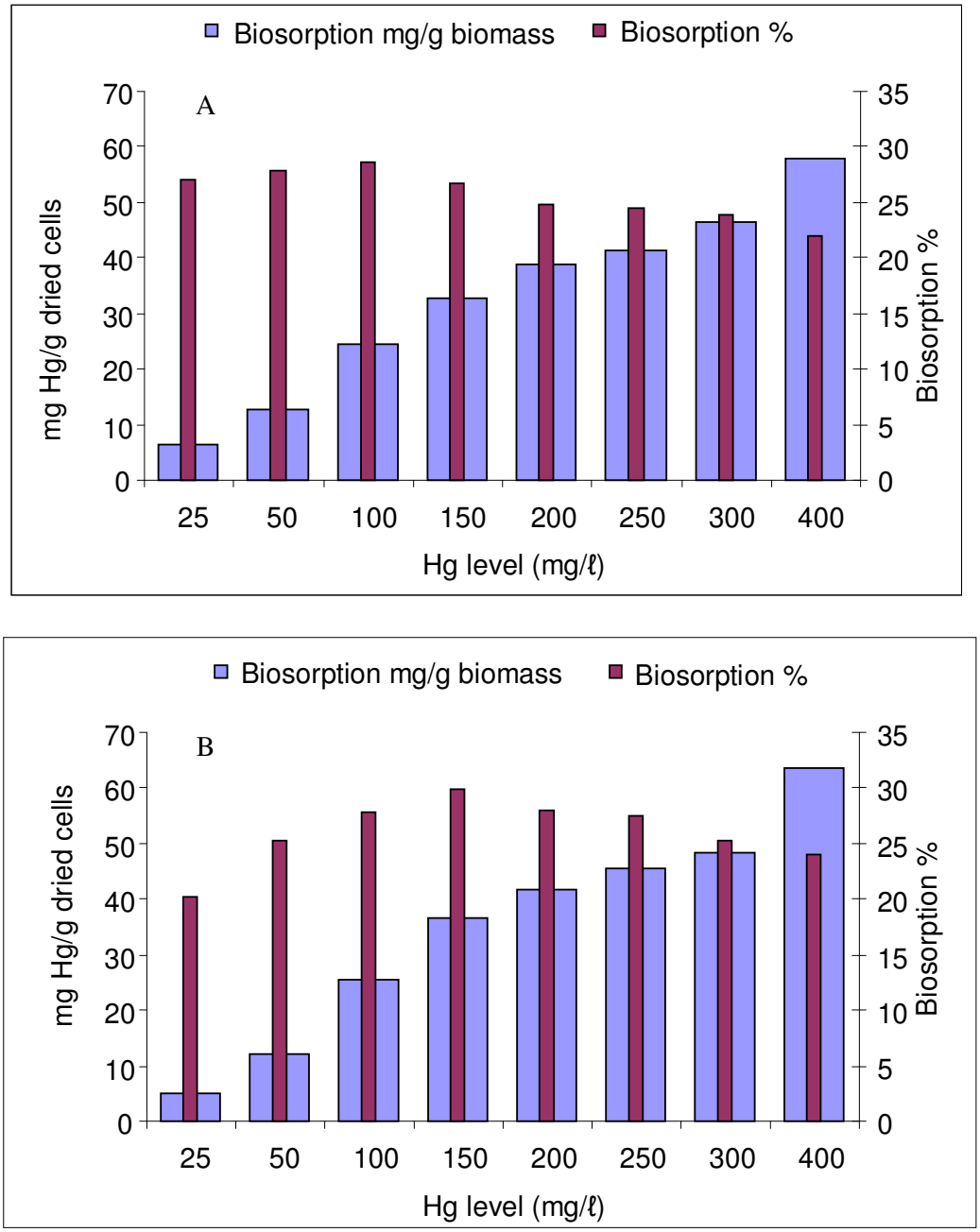

Figure 2. Effect of initial mercury level on biosorption of mercury (mg Hg/g dry cells and as %) by dry biomass of *K. pneumoniae* (A) at pH 5 and *P. aeruginosa* (B) at pH 5.8.

Selatnia et al., 2004).

Biosorption time

The data of mercury uptake (0.1 g Hg/L) at the optimum pH 5 (for *K. pneumoniae*) and pH 5.8 (for *P. aeruginosa*) with 1 g dried cells/L (Figure 4) showed that the rapid uptake of mercury occurred in the first 5 min, accounting for about 6.17 mg Hg/min for *K. pneumoniae* and about 8.87 mg Hg/min for *P. aeruginosa*. This probably is due to the availability of sorption sites at the beginning of the experiments, which is occupied suddenly by the mercuric ions from the solutions (Green-Ruiz, 2006). The results showed that time required for attaining equilibrium was less than 60 min, where biosorption of Hg mg/min dropped markedly (about 72 and 69% for *K. pneumoniae* and *P. aeruginosa*, respectively) as the contact time extended from 5 to 60 min. It is known that the rate of metal uptake is influenced by factors affecting mass

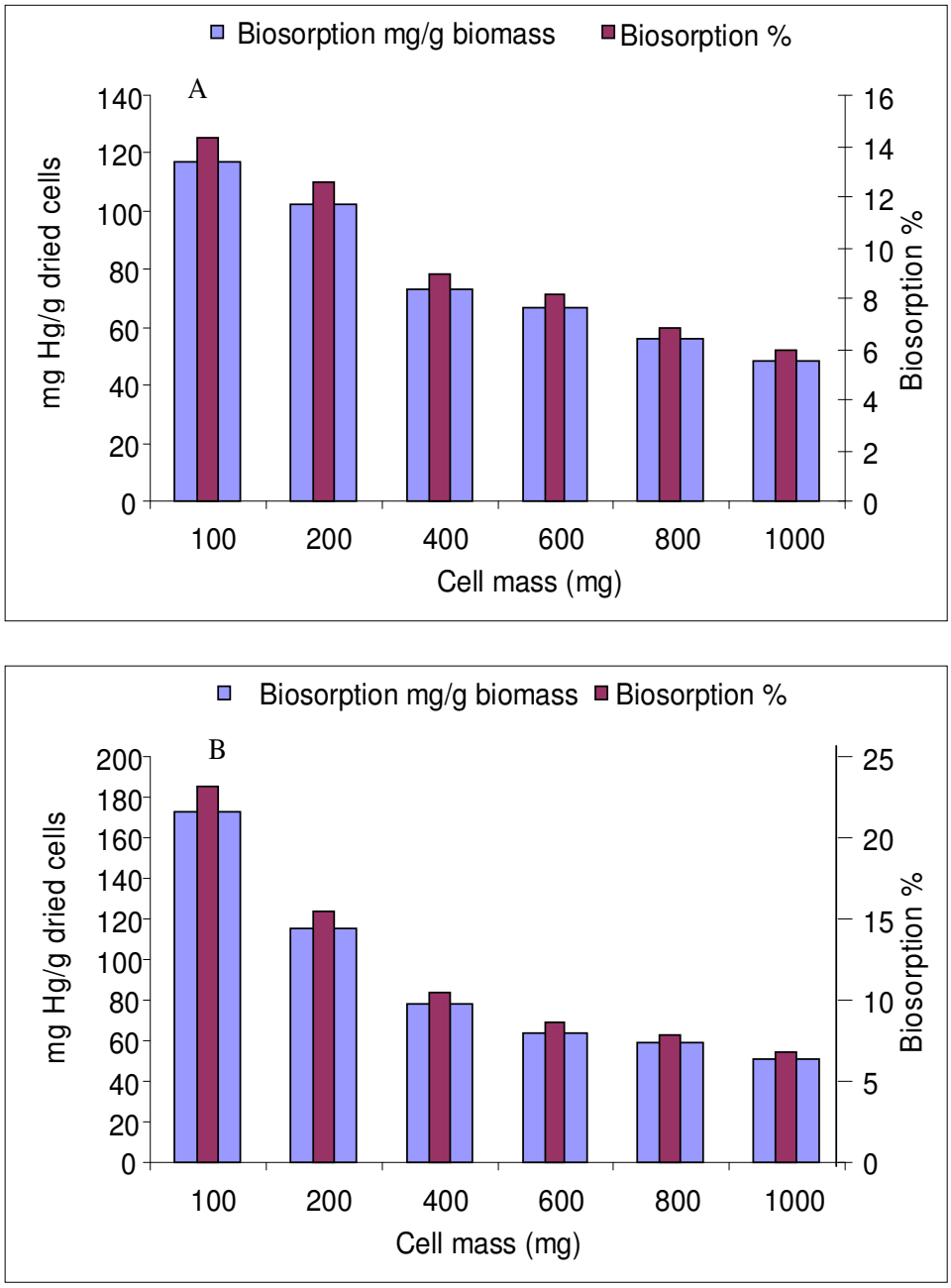

Figure 3. Effect of dried powdered cells level on biosorption of mercury (mg Hg/g dried cells and as %) by *K. pneumoniae* (A) at pH 5 and *P. aeruginosa* (B) at pH 5.8.

transfer from bulk solution to binding sites. It was indicated that various steps are involved in the transfer of metal from bulk solution to binding sites (Weber, 1995). First is the bulk transport of metal ions in solution phase, which is usually rapid because of mixing and adaptive flow (Gadd, 1988). Second, film transport involves diffusion of metal through a hydrodynamic boundary layer around the biosorbent surface, and third, actual adsorption of metal ions by active sites of the biomass is considered to be rapid and equivalent to an equilibrium reaction (Weber, 1985). In the case of mercury biosorption by the tested bacteria, the experimental conditions allowed a normal mixing of solutes and biomass (dry cells) in the system that partially suppressed the kinetic limitations of the first and second steps and hence equilibrium was attained at less than 60 min. Therefore, the kinetics of the process was influenced by the three steps. Similarly, it was reported that biosorption of mercury by *Bacillus* sp. was rapid in the first 20 min and equilibrium was reached around 60 min (Green-Ruiz,

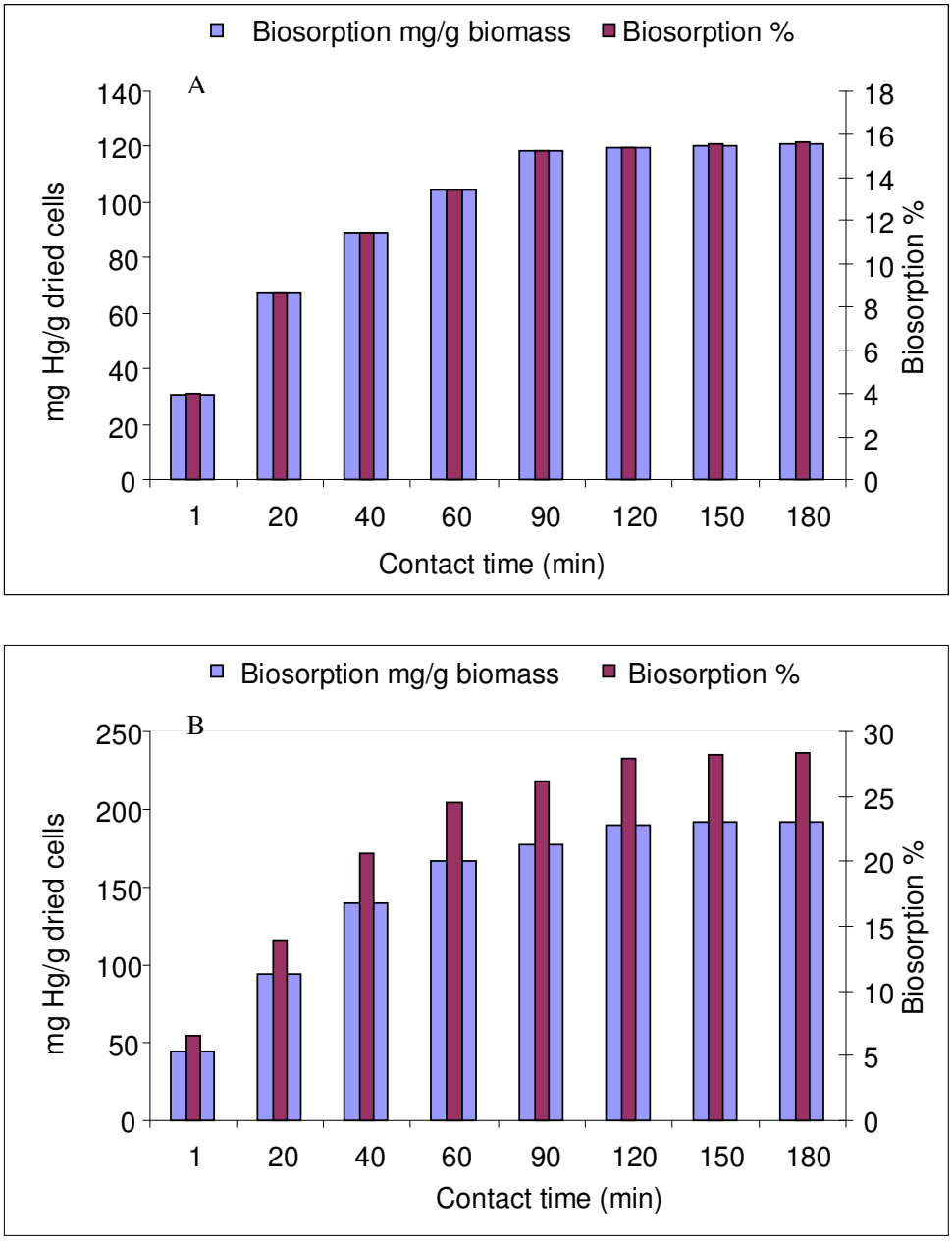

Figure 4. Effect of contact time on biosorption of mercury (mg Hg/g dried cells and as %) by *K. pneumoniae* (A) at pH 5 and *P. aeruginosa* (B) at pH 5.8.

2006). While, Al-Garni (2005) found that biosorption of lead by *Citrobacter freundii* and *K. pneumoniae* was rapid in the first 10 min and equilibrium was attained at less than 70 min. However, it is difficult to compare between those results and the results of this study because of the differences between the different parameters that play a role in the adsorption mechanisms, such as inherent characteristics of the adsorbent organisms (surface area, protein and carbohydrate composition, surface charge capacity), metal affinity and experimental conditions (pH, temperature and sampling periods) (Kacar et al., 2000). The results also indicated that the average of Hg (II) absorption, as mg/min, at the different contact periods of *P. aeruginosa* has 1.5 fold increase as compared to *K. pneumoniae*. This finding correlates with the study's finding that the first bacterium had maximum biosorption of mercury at 150 mg/L, while *K. pneumoniae* attained maximum biosorption at only 100 mg Hg/L. Also, it correlates with the fact that *P. aeruginosa* slime forming Gram -ve bacillus is more efficient to biosorb mercury than capsulated Gram -ve bacillus (*K. pneumoniae*), due to the structural differences between the slime layer

components (contain high negative charge) and the constituents of *K. pneumonia* capsule with low negatively charged components.

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Full Length Research Paper

Improvement of gas chromatographic analysis for organic acids and solvents in acetone-butanol-ethanol fermentation from sweet sorghum juice

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Modern development for gas chromatographic peak recognition of organic acids and organic solvents in acetone-butanol-ethanol (ABE) fermentation was investigated under different temperature programmes. Either propanol or iso-butanol was used as an internal standard. The results showed that short retention time and fair recognition peak of the compounds were obtained under the following column temperature programme: 10 min at 150°C, 15°C/min to 180°C and 20 min at 180°C. The appearance of the chromatograms showed that iso-butanol allowed higher resolution and satisfactory peak shape for the tested compounds than propanol. Calibration curve between ratios of standard peak area per internal standard peak area and concentrations of the organic acids and organic solvents under these conditions had a good linear correlation with $R^2 \geq 0.998$. When ABE fermentation by *Clostridium beijerinckii* JCM1390 from sweet sorghum juice was carried out in a 2-L fermenter, the highest butanol concentration was 7.56 g/l with the butanol yield of 0.33 g/g sugar utilized.

Key words: Acetone-butanol-ethanol fermentation, column temperature programme, gas chromatography, sweet sorghum juice.

INTRODUCTION

Depletion of fossil fuels and fluctuating prices have rekindled and have been interesting in the development of renewable fuels/energy sources such as butanol (Qureshi and Blascheck, 2001). Butanol can be produced from a variety of renewable sources e.g. glucose, whey permeated, corn and cassava (Qureshi and Blascheck, 2001; Thang et al., 2010). This alcohol is widely used in the manufacturing of resin, cleaning fluids, plasticizers and in the action with acids to form esters (Wei et al., 2004). The production of a mixture of acetone, butanol and ethanol fermentation by using the anaerobic bacterium *Clostridium acetobutylicum* continues to receive attention because of its potential commercial significance (Maddox et. al., 1995).

Sweet sorghum [*Sorghum bicolor* (L.)] has been promised as a large scale energy crop because its stalks contain high fermentable sugar and it can be cultivated in nearly all temperatures and tropical climate areas (Sree et al., 1999; Laopaiboon et al., 2009). It is also one of the most drought resistant agricultural crops because of its capacity to remain dormant during the driest periods (Woods, 2000). The juice from sweet sorghum stalk can be efficiently converted into a biofuel, ethanol (Laopaiboon et al., 2009). However, there has been no report on using sweet sorghum juice as a substrate for butanol production.

A fermentation process producing acetone, butanol and ethanol has been known as acetone-butanol-ethanol (ABE) fermentation. ABE-producing clostridia possess two distinct characteristic phases in energy acquiring

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Abbreviations: ABE, Acetone-butanol-ethanol; **GC,** gas chromatography; **FID,** flame ionization detector; **CMM,** cooked meat medium; **OFN,** oxygen free nitrogen; **TGY,** tryptone-glucose-yeast extract; **DNS,** dinitrosalicylic acid.

pathway, that is, acidogenesis and solventogenesis (Jones and Woods, 1986). During acidogenesis, cell growth is exponential and products are acetic and butyric acids with ATP formation. In solventogenesis, cell growth enters stationary phase, the organic acids from the acidogenesis are reutilized and acetone, butanol and ethanol are produced (Tashiro et. al., 2004). Therefore, it is necessary to identify and monitor concentrations of the organic acids and the organic solvents during fermentation process.

Most researchers have used gas chromatography or GC for acid and solvent analysis from ABE fermentation under various conditions and most of them used nitrogen as a carrier gas. Gapes et al. (1996) analyzed product concentrations with a glass column (inside diameter of 3.2 mm, length of 2.6 m) packed with Chromosorb 101 at a column temperature of 170°C and flame ionization detector (FID). Assobhei et al. (1998) used a 2-m long glass column packed with Porapak Q l00/120 mesh equipped with FID. The injector and detector temperatures were 220°C, the column temperature was programmed from 175 to 225°C. Montaya et al. (2000) found that the solvents were separated in a steel column packed with Chromosorb 102. The injector and detector temperatures were 200°C. The column temperature was programmed from 200 to 210°C, at a rate of 1°C/min. The injector and detector temperatures were 260°C. Qureshi et al. (2001) used GC equipped with FID and a capillary (0.53 mm, 30 m). The initial and final column temperatures were 60 and 160°C, respectively. Tashiro et al. (2004) determined ABE products of ABE fermentation by using GC equipped with FID and a 15 m capillary column. The column temperature was programmed from 50 to 170°C at the rate of 10°C/min. The injector and detector temperatures were 250°C and helium was used as a carrier gas. The two internal standards normally used for ABE analysis are propanol (Montaya et al., 2000; Qureshi et al., 2001) and iso-butanol (Gapes et al., 1996; Assobhei et al., 1998; Tashiro et al., 2004).

Although, GC conditions for acid and solvent quantification have been investigated by a number of researchers, product analysis by GC is also dependent on several parameters such as GC instrument, type of packing material in column, inside diameter and length of column, etc. Thus, the aim of this work is to develop a method to accurately detect the main products in the ABE fermentation broth, that is, acetic acid, butyric acid, acetone, butanol and ethanol using GC under different column temperature programmes. ABE fermentation from sweet sorghum juice by *C. beijerinckii* JCM1390 was also investigated.

MATERIALS AND METHODS

GC analysis

Standard solution for GC analysis consisted of acetic acid (Sigma-Aldrich, Germany), 20.0 g/l; acetone (Merck, Germany), 10.0 g/l; butyric acid (BDH, UK), 10.0 g/l; butanol (BDH, UK), 10.0 g/l; ethanol (BDH, UK), 10.0 g/l of 20% (v/v) ortho-phosphoric acid (BDH, UK). The standard mixture was diluted at different concentrations by using the ortho-phosphoric acid as a diluent. Propanol (BDH, UK) at 8.0 g/l of 20% (v/v) ortho-phosphoric acid was used as an internal standard. The standard mixtures at various concentrations were mixed with the internal standard at the ratio of 1:1 (v/v). Then, 1 µl of the mixtures was injected at least three times into GC to ensure repeatability. The standard calibration curve between concentrations of the standard mixtures and the ratios of peak area (standard peak area per internal standard peak area) was established. Iso-butanol (BDH, UK) at concentration of 8.0 g/l (in 20% ortho-phosphoric acid) was also used as an internal standard. The standard mixtures at various concentrations were mixed with this internal standard as described for propanol.

GC analysis of organic acids and organic solvents was performed using a GC-14B (Shimadzu, Japan) gas chromatograph in a splitless mode. Separation took place in a 2-m long and 2-mm inner diameter stainless steel column packed with Parapack Q, 80/100 mesh (Resteck, USA). The injector was operated at 220°C and the FID was kept at 230°C. Nitrogen gas was used as a carrier gas at a pressure of 150 kPa. The column temperature was varied under five different column temperature programmes as stated in (A) - (E): (A) 145°C (isothermal programme); (B) 150°C (isothermal programme); (C) 10 min at 150°C, 10°C/min to 180°C, 20 min at 180°C; (D) 10 min at 150°C, 15°C/min to 180°C, 20 min at 180°C; (E) 10 min at 150°C, 15°C/min to 180°C, 5 min at 180°C, 10°C/min to 200°C, 20 min at 200°C. Chromatographic data were acquired by C-R7A (Shimadzu, Japan).

ABE fermentation

Microorganism and inoculum preparation

C. beijerinckii JCM 1390 was maintained as spore suspension and stored at 4°C in sterile distilled H_2O. The spore suspension (0.5 ml) was heat shocked at 80°C for 10 min, cooled in iced-water for 1 min (Areesirisuk et al., 2006), and inoculated into 20 ml sealed serum bottle containing 10 ml cooked meat medium (CMM). The culture was incubated at 37°C until growth was observed (16 - 19 h). Before the inoculation, CMM in the bottle was rapidly sparged with filtered oxygen free nitrogen (OFN) gas to create a strictly anaerobic condition. Five ml of CMM cell suspension, which was highly motile cells, were transferred into tryptone-glucose-yeast extract (TGY) medium and incubated at 37°C for 8 h. This culture was used as an inoculum for butanol production.

Raw material and medium preparation

Sweet sorghum juice cv. KKU40 modified from cv. Keller was obtained from the Department of Agronomy, Faculty of Agriculture, Khon Kaen University, Thailand. After extraction, the juice was kept at -18°C until use.

The sweet sorghum juice was supplemented with 1 g/l of yeast extract (Oxoid, UK) (Qureshi and Blaschek, 1999) and pH of the juice was adjusted to 5.5 with 1.0 N NaOH (BDH, UK). The juice was then transferred into a 2-L fermenter (B. Braun Biotech International, Biostat B, Germany) with a final working volume of 1.5 L and autoclaved at 110°C for 25 min. Three stock solutions (A, B and C) were prepared (Qureshi and Blaschek, 1999). The stock solution A was composed of K_2HPO_4 (BDH, UK), 50 g/l; KH_2PO_4 (BDH, UK), 50 g/l and ammonium acetate (BDH, UK), 220 g/l. The stock solution B was composed of para-amino-benzoic acid (BDH, UK), 0.1 g/l; thiamine (BDH, UK), 0.1 g/l and biotin (Fluka, Switzerland), 0.001 g/l. The stock solution C was composed of $MgSO_4 \cdot 7H_2O$ (Riedel-DeHaen, Germany), 20 g/l; $MnSO_4 \cdot H_2O$ (BDH, UK), 1 g/l; $FeSO_4 \cdot 7H_2O$ (Riedel-DeHaen, Germany), 1 g/l

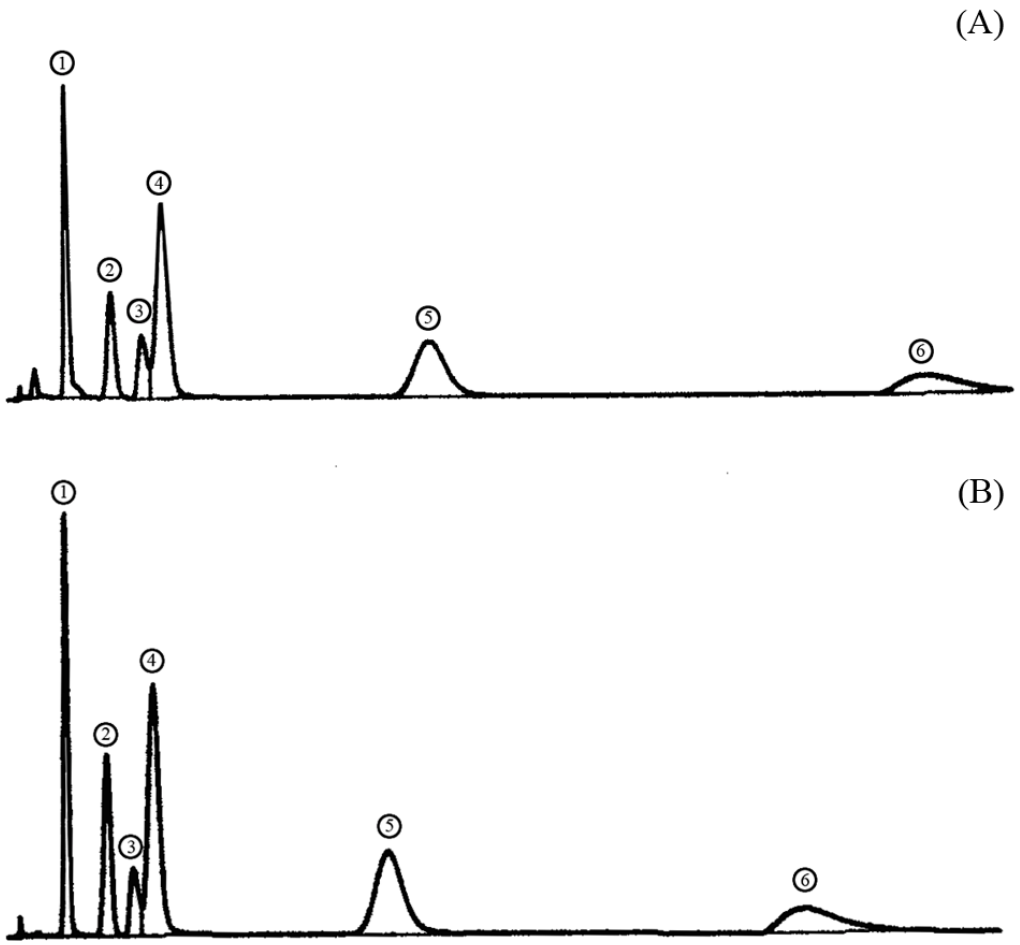

Figure 1. GC chromatogram of organic acid and organic solvent standards under isothermal temperature programme. A, Condition A (isothermal programme at 145°C); B, condition B (isothermal programme at 150°C); ①, ethanol; ②, acetone; ③, acetic acid; ④, propanol; ⑤, butanol; ⑥, butyric acid.

and NaCl (BDH, UK), 1 g/l. The three stock solutions were sterilized by filtration through a 0.2 µm pore-size cellulose acetate membrane filter before adding (15 ml each) into the sterile juice.

Fermentation conditions and analyses

OFN gas was quickly flushed into the sterile medium in the fermenter to create a strictly anaerobic condition before the *C. beijerinckii* JCM 1390 in TGY medium (5%, v/v) was inoculated. The fermentation was operated at the agitation rate of 50 rpm, 37°C and OFN gas was flushed continuously across the surface of the medium at the rate of 0.2 ml/min until gases in the fermentation broth were generated by visual observation. The samples were withdrawn at time intervals for analysis.

Acid and solvent concentrations of the supernatant were analyzed by GC under the optimum conditions obtained. Total sugar and reducing sugar concentrations of the supernatant were determined by phenol-sulphuric acid and dinitrosalicylic acid (DNS) method, respectively (Scherz and Bonn, 1998). The butanol yield ($Y_{p/s}$) and volumetric butanol productivity (Q_p) were calculated by the following equations:

$$Y_{p/s} = \frac{P}{TS}$$

$$Q_p = \frac{P}{t}$$

Where, P is the butanol concentration (g/l), TS is the total sugar utilized (g/l) and t is the fermentation time (h).

RESULTS AND DISCUSSION

Gas chromatographic peck identification

Isothermal programme

Figure 1 shows the chromatogram of organic acid and organic solvent analysis under isothermal temperature programme and propanol as an internal standard. Under condition A (145°C), all peaks of the standards could be

Table 1. Retention time of organic acids and organic solvents under different GC conditions.

GC condition*	Retention time (min)						
	Ethanol	Acetone	Acetic acid	Propanol	Iso-butanol	Butanol	Butyric acid
A with propanol**	3.890	6.774	8.769	10.113	-	27.359	59.491
B**	3.513	5.983	7.549	8.801	-	22.983	48.071
C**	3.477	5.929	7.474	8.723	-	16.201	23.612
D**	3.516	5.999	7.583	8.830	-	15.961	23.421
E**	3.480	5.940	7.500	8.743	-	15.875	21.028
D with iso-butanol***	3.782	6.174	8.760	-	15.029	16.712	24.844

*Condition A: Isothermal programme at 145°C; condition B: isothermal programme at 150°C; condition C: 10 min at 150°C, 10°C/min to 180°C and 20 min at 180°C; condition D: 10 min at 150°C, 15°C/min to 180°C and 20 min at 180°C and condition E: 10 min at 150°C, 15°C/min to 180°C, 5 min at 180°C, 10°C/min to 200°C and 20 min at 200°C. **, ***propanol or iso-butanol as the internal standard, respectively.

separated, but butanol and butyric acid peaks had band broadening and the retention time of the last peak was too long (Figure 1A). To make the last two peaks sharper and reduce analysis time, column temperature was increased from 145 to 150°C (condition B). The results indicated that retention time of the standard mixtures was decreased with increasing column temperature (Figure 1B and Table 1). Nonetheless, butanol and butyric acid peaks still had band spreading. Thus, column temperature programme conditions were applied.

Temperature programme

In order to reduce retention time and alleviate band broadening, column temperature programme under condition C (10 min at 150°C, 10°C/min to 180°C and 20 min at 180°C) was tested. The results indicated that retention time of the standard mixtures was significantly decreased (Figure 2A). In particular, the retention time of butanol and butyric acid peaks were reduced to approximately 10 and 51%, respectively, when compared to those under condition B. This suggested that temperature programme could improve chromatogram for the standard mixture separation. However, a tailing peak of butyric acid was observed. To develop the butyric acid peak, rate of increasing column temperature was adapted to condition D (10 min at 150°C, 15°C/min to 180°C and 20 min at 180°C). The chromatogram and retention time of ethanol, acetone, acetic acid, propanol, butanol and butyric acid under condition D (Figure 2B) were similar to those under condition C (Figure 2A). This implied that chromatographic separation could not always be improved by increasing accelerating rate of column temperature only.

To alleviate the tailing peak of butyric acid, the column temperature programme of condition E (10 min at 150°C, 15°C/min to 180°C, 5 min at 180°C, 10°C/min to 200°C and 20 min at 200°C) was operated (Figure 2C). Under condition E, the retention time of butyric acid peak was decreased by 56% when compared with that under isothermal condition B, whereas those of ethanol, acetone, acetic acid, propanol and butanol were still similar to those under condition C and D (Table 1). However, the baseline of chromatogram under condition E was not good enough. Therefore, it was not selected for further studies.

Under condition D, the peaks of acetic acid and propanol were overlapped and the acetic acid peak was closed to the acetone peak (Figure 2B). Therefore, another internal standard normally used, iso-butanol (Gapes et al., 1996; Assobhei et al., 1998; Tashiro et al., 2004), was tested to improve the chromatogram. The appearance of the chromatogram demonstrated that iso-butanol allowed high resolution and satisfactory peak shape for the tested compounds (Figure 3). Analysis of the chromatogram using propanol compared with that using iso-butanol revealed that the organic acids and solvents under using the latter internal standard were separated better than those using the former one (Figures 2B and 3). Therefore, the column temperature programme under condition D and iso-butanol as an internal standard were chosen to evaluate calibration curves of standard mixtures for ABE production.

The standard mixtures at various concentrations were injected into the GC under condition D. The calibration curves between ratios of standard peak area per internal standard (iso-butanol) peak area and concentrations of the organic acids and organic solvents were established (Figure 4). The results showed that the ratios of standard peak area per internal standard peak area and concentration of the standards had a good linear correlation with $R^2 \geq 0.998$. Under condition D, the sensitivity or detection limit of ethanol, acetone, acetic acid, butanol and butyric acid were 0.31, 0.31, 1.25, 0.63 and 0.63 g/l, respectively.

ABE fermentation from sweet sorghum juice

Batch ABE fermentation of *C. beijerinckii* JCM 1390 was performed to evaluate the GC condition developed and to study the possibility of the use of the sweet sorghum juice

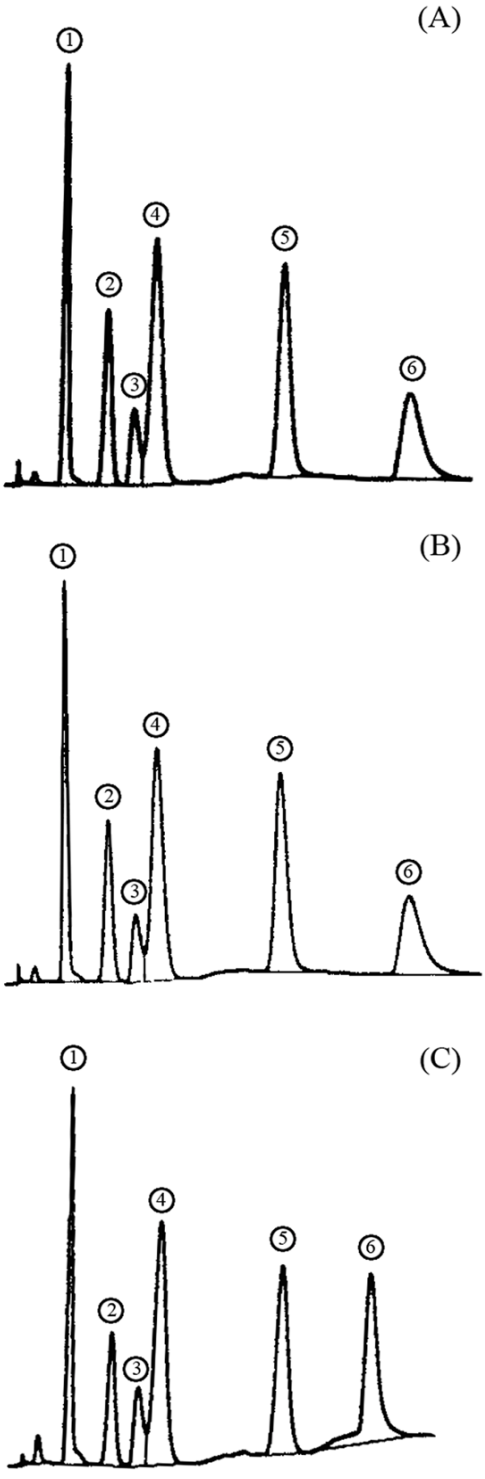

Figure 2. GC chromatogram of organic acid and organic solvent standards under column temperature programme. A, Condition C (10 min at 150℃, 10℃/min to 180℃ and 20 min at 180℃); B, condition D (10 min at 150℃, 15℃/min to 180℃ and 20 min at 180℃); C, condition E (10 min at 150℃, 15℃/min to 180℃, 5 min at 180 ℃, 10℃/min to 200℃ and 20 min at 200℃); ①, ethanol; ②, acetone; ③, acetic acid; ④, propanol; ⑤, butanol; ⑥, butyric acid.

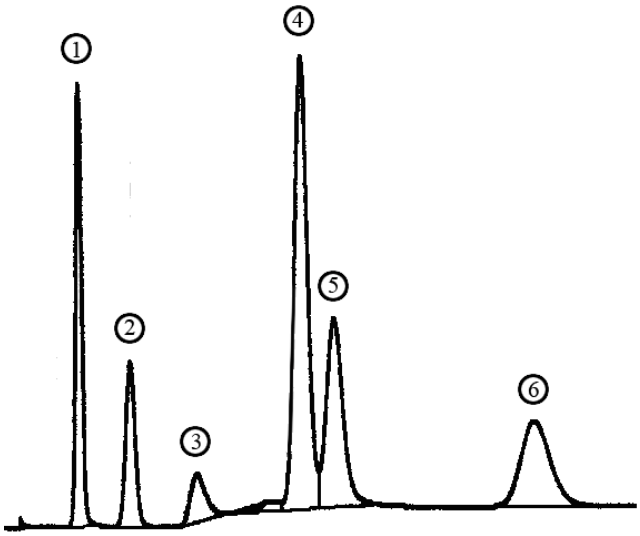

Figure 3. GC chromatogram of organic acid and organic solvent standards under condition D (10 min at 150℃, 15℃/min to 180℃ and 20 min at 180℃) using iso-butanol as an internal standard. ①, Ethanol; ②, acetone; ③, acetic acid; ④, iso-butanol; ⑤, butanol; ⑥, butyric acid.

as a substrate for butanol production. The profiles of total sugar, reducing sugar, pH, organic acids and organic solvents during fermentation are shown in Figure 5. Total sugar and reducing sugar were decreased in the first 75 h. After that, their concentrations rarely changed. The total sugar and reducing sugar utilized were similar with the values of 24.91 and 20.19 g/l, respectively, indicating that almost all sugar utilized were glucose and fructose because they were the main reducing sugar in the sweet sorghum juice (Laopaiboon et al., 2009). When the products from the fermentation were monitored (Figure 5), the results showed that two distinct characteristic phases, acidogenesis and solventogenesis, occurred.

Acidogenesis phase of *C. beijerinckii* JCM 1390 had occurred since the beginning of the fermentation. Acetic acid concentration was increased to 2.89 g/l at 38 h and slightly decreased after that, while butyric acid concentration was increased to 2.45 g/l at 38 h and was highest (3.05 g/l) at 95 h. The accumulation of the acetic and butyric acids resulted in a decrease in pH of the broth from 5.51 at the beginning to 4.98 at 38 h. pH of the broth was slightly increased after that and remained constant at 5.13. Similar results were observed by Wang et al. (2005) who studied ABE production by *C. beijerinckii* NCIMB 8052 grown on TGY medium containing 0.5% (w/v) glucose at 37℃. They reported that pH of the broth was decreased with an increase in organic acids.

Soventogenesis phase was observed after approximately 20 h of the fermentation (Figure 5). Butanol concentration was rapidly increased after 38 h and the highest value was obtained at 95 h. Ethanol and acetone were produced at very low levels with the maximum

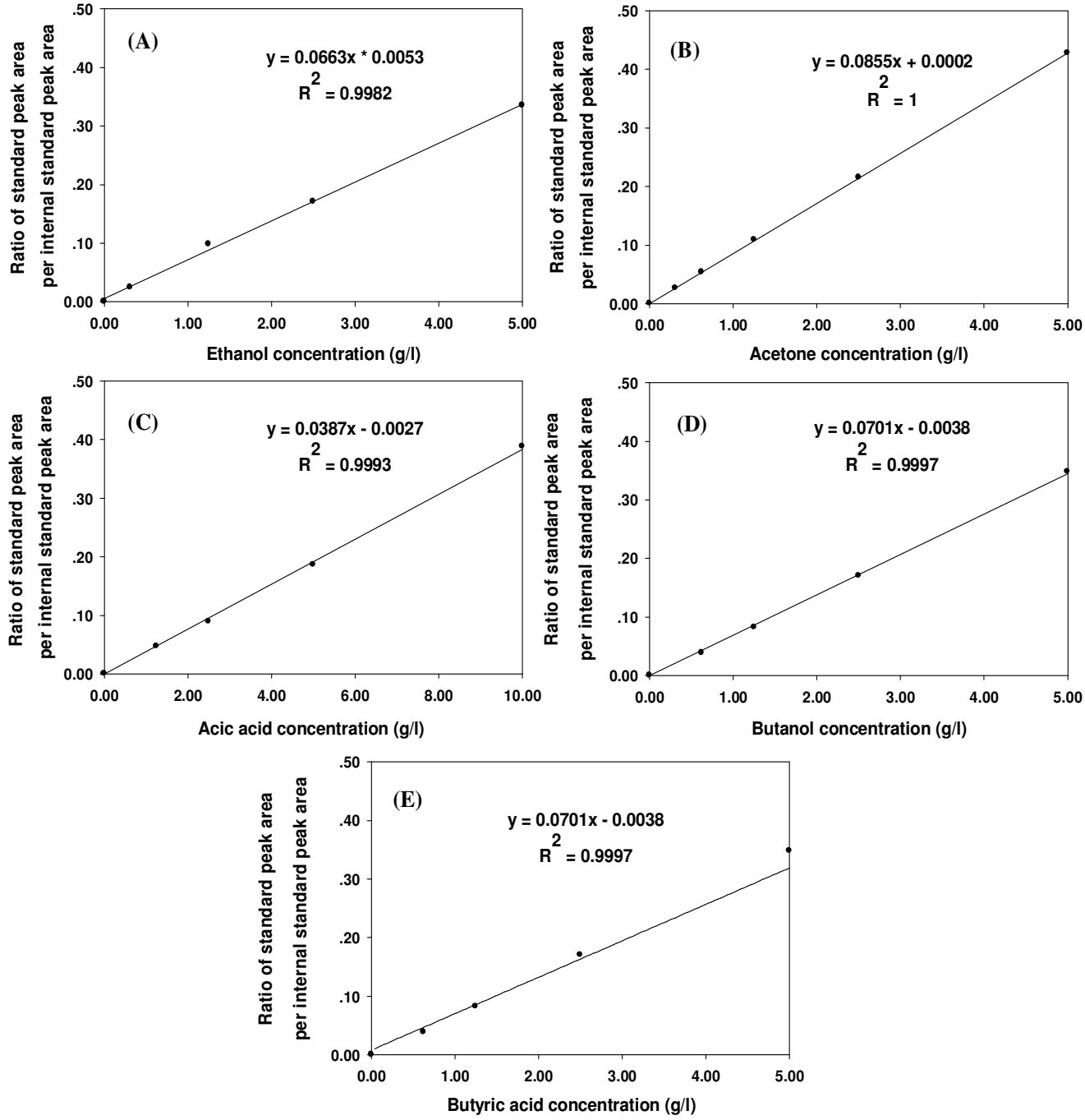

Figure 4. Calibration curves of ethanol (A), acetone (B), acetic acid (C), butanol (D) and butyric acid (E) using iso-butanol as an internal standard.

values of 0.72 and 0.66 g/l, respectively. *C. beijerinckii* JCM 1390 produced butanol much higher than ethanol and acetone. The highest butanol concentration, $Y_{p/s}$ and Q_p were 7.56 g/l, 0.30 g/g and 0.08 g/l/h, respectively. The results obtained from this study were satisfactory in terms of very low ethanol and acetone produced when compared to those of Tsuey et al. (2006) in which the acetone concentration was about 60% of butanol concentration.

Conclusions

GC conditions have been developed to improve the gas chromatographic peak and retention time of the main

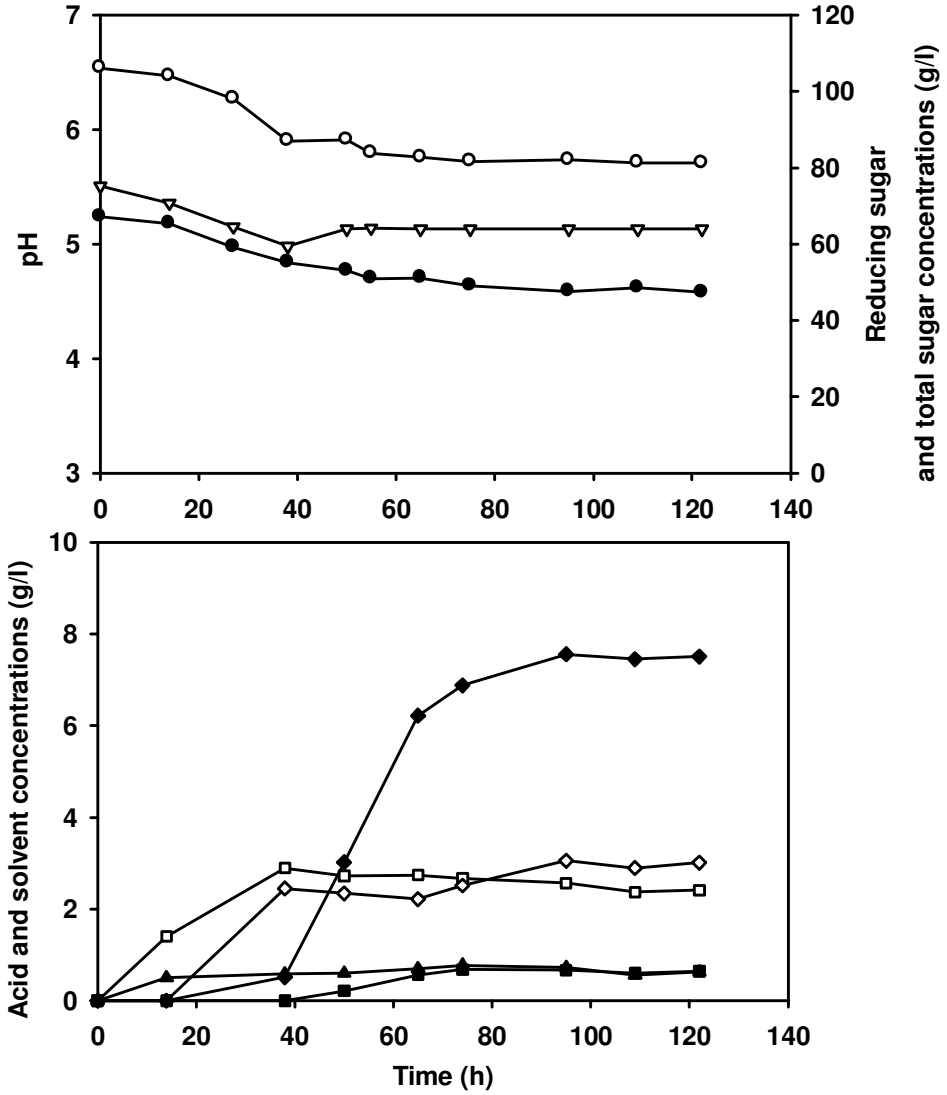

Figure 5. Batch culture profiles and product formation of *C. beijerinckii* JCM 1390 from the sweet sorghum juice. ∇, pH; ●, reducing sugar; ○, total sugar; ■, acetone; ♦, butanol; ▲, ethanol; □, acetic acid; ◊, butyric acid.

organic acids and organic solvents produced in ABE fermentation under different temperature programmes. The optimum conditions for identification and monitoring of ABE concentration during fermentation process of the acids and the solvents using GC were 10 min at 150°C, 15°C/min to 180°C and 20 min at 180°C and iso-butanol was used as an internal standard. The ratio of standard peak area per internal standard peak area and concentrations of the standards had a good linear correlation with $R^2 \geq 0.998$. The ABE fermentation of C. *beijerinckii* JCM 1390 demonstrated that sweet sorghum juice could be used as a substrate for butanol production. However, low butanol production rate or in our study needs to be improved. The effects of initial pH and total sugar to improve the rate of butanol production from sweet sorghum juice are under investigated.

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Full Length Research Paper

Anti-tumor activity of triterpenoid-rich extract from bamboo shavings (*Caulis bamfusae* in Taeniam)

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Bamboo shavings are a kind of Chinese traditional medicine, which have been certificated as a material of functional food by the Ministry of Health in China. The anti-tumor activities of a triterpenoid-rich extract of bamboo shavings (EBS) and its main component, friedelin were evaluated in the present study. It was proved that EBS could inhibit the growth of P388 and A549 cancer cell lines effectively by SRB and MTT assay. Meanwhile, EBS had notable inhibitory effect on the sarcoma-loaded mice S180 model, which led to a depressed trend of tumor weight, compared to cyclophosphamide. Furthermore, the anti-tumor activity of friedelin monomer, a main triterpenoid separated from EBS, was tested by MTT assay and results showed that friedelin displayed rather strong anti-tumor activities on the proliferation of four cancer lines, A375, L929, Hela and THP-1, with a time-dose relationship compared to de-methyl-cantharidin, respectively. Therefore, it is suggested that EBS has a great potential to be applied in functional food for its anti-tumor activity, in which friedelin was one of the most important active factors.

Key words: Extract of bamboo shavings, anti-tumor, friedelin, triterpenoid (*Caulis bamfusae* in Taeniam).

INTRODUCTION

Bamboo shavings (*Caulis Bamfusae* in Taeniam), which are the intermediate layer of the stems of *Bambusa tuldoides* Munro, *Sinocalamus beecheyanus* var. *pubescens* P. F. Li or *Phyllostachys nigra* (Lodd.) Munro var. *henonis* (Mitf.) Stapf ex Rendle, are a perennial plant of *Gramineae* family in plant kingdom. Bamboo shavings can be obtained by scraping off the coat from bamboo stems, cutting the stems into slices and binding them together by drying in shadowy places. They have been used as a clinical Chinese traditional medicine mainly to lessen or cure stomach ache, diarrhea and vomiting, chest diaphragm inflammation, restlessness and excessive thirst and its efficacy had been recorded in the material media of past dynasties in Chinese history.

It is well known that there are abundant biological active compounds in bamboo shavings such as triterpenoids, saponins and sterols. Triterpenoids and their saponins, which are important biological active components in natural kingdom, can be divided into triterpenoid saponins and steroid saponins according to their different chemical structures (Yao, 2001). The triterpenoid-rich extract from Chinese bamboo shavings (EBS), which mainly contains a group of pentacyclic triterpenoids such as friedelin, friedelinol, lupenone and lupenol, etc (Figure 1), is a low polar component extracted from bamboo shavings using the CO_2 supercritical fluid extraction (SFE) technique.

Many researchers have reported that triterpenoids have many physiological functions such as anti-sepsis, anti-virus, anti-tumor, anti-fatigue, anti- hyperlipidemia, anti-hypertension, anti- hyperglycemia, etc (Yao, 2001). Research data about triterpenoids published in many countries refer to their prominent physiological functions especially on anti-tumor.

The reports on the biological activities of triterpenoids

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Abbreviations: EBS, Extract of bamboo shavings; **TBP,** triterpenes of betula platyphylla; **SRB,** Sulforhodamine B; **MTT,** microculture tetrazolium; **SFE,** supercritical fluid extraction; **IC_{50},** half-maximal inhibition; **FCS,** fetal calf serum; **HCPT,** hydroxycamptothecine; **DMSO,** dimethyl sulfoxide; **GC-MS,** gas chromatography-mass spectrometry; **EBV-EA,** Epstein-Barr virus early antigen; **TBP,** triterpenes of betula platyphylla.

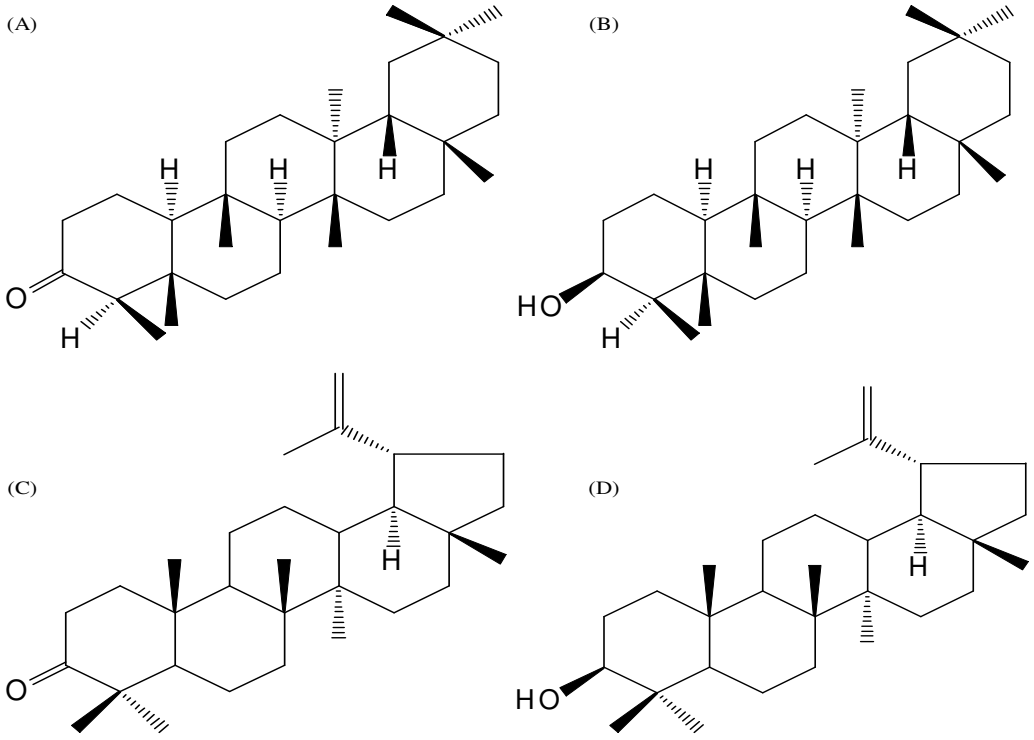

Figure 1. Chemical structure of mainly triterpenoids in EBS. (A) Friedelin, (B) Friedelinol, (C) Lupenone AND (D) Lupenol.

in bamboo shavings and physical data on the chemical composition are limited. In the preliminary tests, it was shown that EBS and friedelin have great potential for its anti-fatigue activity (Zhang et al., 2006), anti-hyperlipidemic activity and anti-hypertensive activity (Jiao et al., 2007). On the other hand, many researchers have reported the anti-tumor activity of various triterpenoids and their derivatives (Fukuda et al., 2005, 2006). However, there was no systematic report to investigate anti-tumor studies on the triterpenoid-rich extract from Chinese bamboo shavings, especially its main component, friedelin.

In the present study, the filtration tests of EBS and its mainly active monomer, friedelin, *in vitro* with the research objects of mouse leukemia (P388) cells, human lung adenocarcinoma (A549) cells, human malanoma (A375) cells, mouse lung epithelial tumor (L929) cells, human cervical tumor (Hela) cells, human macrophage tumor (THP-1) cells and sarcoma-loaded mice S180 model were systematically discussed so that the anti-tumor activities of EBS and friedelin were primarily evaluated.

MATERIALS AND METHODS

Plant material

Bamboo shavings were collected from the intermediate layer of the stems of *Phyllostachys nigra* var. *henonis* in Anji District (Huzhou, Zhejiang, China). This sample was authenticated by the Research Institute of the Subtropical Forestry of Chinese Academy of Forestry (Hangzhou, China) (Zhang et al., 2004). A voucher specimen EBS was deposited at the Herbarium of the Laboratory of Research and Development of Natural Products in Department of Food Science and Nutrition (Zhejiang University, Hangzhou, Zhejiang, China).

Preparation of EBS and identification of triterpenoids

Triterpenoid-rich extract was prepared from bamboo shavings (*P. nigra* var. *henonis*) identified by Research Institute of Subtropical Forestry of the Chinese Academy of Forestry (Hangzhou, China). Briefly, fresh bamboo shavings were adequately washed with water and dried in the air. The coarse powder of bamboo shavings was obtained after comminution and filtration (10 ~ 20 mesh). 5.5 kg powder was circularly extracted using the carbon dioxide SFE technique. Finally, the extract of bamboo shavings (EBS) was collected. Results showed that EBS was a kind of yellow or yellowish green powder with the melting point range from 74 to 79°C and its carbon dioxide SFE yield was about 2%. The main triterpenoids in EBS were identified by GC-MS (Hewlett- Packard 6890 GC and MS Engine 5973 mass spectrometer, Agilent Inc., USA) and analyzed by GC (Agilent 6890N and FID detector, Agilent Inc., USA) (Yao et al., 2004). The chromatographic separation was performed with a HP5-MS column (30 m × 0.25 mm i.d.) coated with a phenyl-methyl polysiloxane stationary phase (film thickness: 0.2 µm). Helium was used as a carrier gas at a flow rate of 1 mL/min. 1 µL EBS sample dissolved in dichloromethane was injected using a splitless injection mode with the injection port temperature at 280°C. The column temperature was held at 100°C for 2 min and then programmed from 100 to 270°C at a rate of 20°C /min, holding for 20 min. The mass selective detector was operated in the electron impact ionization mode with ionization energy of 70

eV. The ion source temperature was 230°C. Full scan mode was used with the MS scan range from 18 to 500 m/z. Data were analysed by Database NIST 98.

Anti-tumor active filtration of EBS *in vitro*

Filtration methods

Sulforhodamine B (SRB) protein staining method were used to evaluated the growth inhibitory effect of the adherent tumor cells (P388 mouse leukemia cells) by EBS and microculture tetrozolium (MTT) reducing method were used to evaluated the growth inhibitory effect of the suspended tumor cells (A549 human lung adenocarcinoma cells) by EBS, respectively. The effect time was 48 and 72 h compared to the control, Vincristin, respectively.

SRB assay

SRB assay of inhibitory effect by EBS was carried out according to the programs by Xiao et al. (2001). Briefly, adherent cells in 0.1 mL media were plated in each well of 96-well plates and allowed to attach for 24 h. EBS was added to the wells to produce the desired final concentrations and the plates were cultured at 37°C for 72 h. Cells were then fixed by gentle addition of 100 µL of cold (4°C) 10% trichloroacetic acid to each well. Plates were washed with deionised water five times and allowed to air dry. Cells were then stained by addition of 100 µL SRB solution [0.4% SRB (w/v) in 1% acetic acid (v/v)] to wells for 15 min. Following staining, plates were quickly washed five times with 1% acetic acid to remove any unbound dye and allowed to air dry. Bound dye was solubilized with 10 mmol/L Tris base (pH 10.5) prior to reading the plates. The optical density (OD) was read on a plate reader at a wavelength of 515 nm with Molecular Devices (model # VERSAmax). The inhibitory effect of HCPT on cultured cells was expressed as IC_{50}. Finally, the mean IC_{50} was calculated according to the data from three replicate tests.

MTT assay

Stock solutions of complexes were freshly prepared in 10% DMSO and diluted to the required concentration with culture when used. Tumor cells were grown in RPMI 1640 medium supplemented with 10% freshly inactivated fetal calf serum (FCS) and antibiotics. The cells harvested from exponential phase (2×10^5 per mL) were seeded equivalently into a 96-well plate. Then the compounds studied were added in a concentration gradient and the final concentrations were maintained at 1000, 100, 10, 1 and 0.1 µM, respectively. The plates were kept at 37°C in a humidified atmosphere of 5% CO_2 and cultured for 48 h; then MTT solution of an appropriate concentration (1 mg/mL) was added to each well and the plates were cultured at 37°C for 4 h. The measurements of absorbance of the solutions related to the number of live cells were performed on an ELISA spectrophotometer at 570 nm (Zhou et al., 2001).

Effect on tumor- bearing mice S180 model by EBS

S180 sarcoma-loaded mice were killed by cervical dislocation before the experiment and were saturated 8 - 10 min in the benzalkonium bromide solution. Then, several tumor blocks were isolated in asepsis operational condition and were made to be a homogenate mixing with the injection physiological saline according to the proportion of 1: 3 (3 mL physiological brine for each 1 g tumor block). Each experimental mouse was injected with 0.2 mL homogenate solution in axilla endermic tissue after partially disinfecting by ethanol iodine. They were divided into several groups at random 24 h later and were accepted oral administration continuously 10 days (once for each day) as follows: (i) control, water supply, 20 mL/kg (0.4 mL/20g), (ii) positive control, cyclophosphamide supply, 30 mg/kg, ip, CTX, (iii) EBS high dose, 1.6 g/kg, (iv) EBS intermediate dose, 0.8 g/kg and (v) EBS low dose, 0.4 g/kg. Mice were killed next day when stopping oral administration. The body was weighed and the corresponding tumor block was isolated and weighed for each mouse. Finally, the tumor inhibition rate was calculated.

Anti-tumor test of friedelin extracted from EBS

The Silica Gel Column Chromatography and Countercurrent Chromatography preparation techniques were used to obtain friedelin monomer isolated from EBS for the sake of evaluating its anti-tumor activity further. The purity of this monomer determined by GC analysis was 90.5%. MTT method was used to demonstrate the anti-tumor activity of friedelin monomer *in vitro*.

A375, L929, Hela and THP-1 were used for active filtration test in this experiment. Among these cell lines, A375, L929 and Hela were adherent cells while THP-1 was a kind of suspended cell. All cells were maintained in the RPMI-1640 culture medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.2% $NaHCO_3$. These supplements were dissolved in deionized water and 0.2 µm filtration membrane was used for filter sterilization. The fetal calf serum was inactivated 30 min at 56°C prior to an experiment. Cells were cultured in a humidified incubator of 5% CO_2 at 37°C.

A375, L929 and Hela cells in logarithmic growth phases were seeded at a cell density of approximately 5×10^4 cells/mL in 96-well plates (100 µL/well). In addition, THP-1 cell was seeded at a cell density of approximately 1×10^5 per mL because it was smaller than others. Samples were added in suspended cells after culturing 4 h and in adherent cells after culturing 12 h. Friedelin was dissolved in DMSO in advance and then in the culture medium with the ultrasonic treatment, where the final concentration of the solvent in the culture medium was < 0.1% (v/v). Sample was added for 7.5, 15, 30, 60, 120, 240, 480 and 960 µmol/L. Each level had four parallel pores and a negative control. The cells were cultured for 24 and 48 h and then 15 µL/well MTT solvent (5 mg/mL) was added.

After culturing for 48 h, the cells were centrifugated at 1500 rpm for 5 min. The supernatant was removed and 150 µL/well DMSO was added, following by micro-vibrator shocking for 10 min and crystal dissolving completely. The absorbance (OD value) was measured by ELISA spectrophotometer at 492 nm. Consequently, the inhibition rate of cell proliferation and the IC_{50} value of friedelin were counted by Bliss method. The de-methyl-cantharidin was took as a positive control and the inhibition rate of A375, L929 and Hela cells were measured by adding de-methyl-cantharidin (120 µmol/L) for 24 and 48 h. Finally, the corresponding data of friedelin were counted.

RESULTS

Identification of triterpenoids in EBS

Some pentacyclic triterpenoids, such as friedelin, friedelinol, lupenone and lupenol, were identified by GC-MS and the chemical structures of these triterpenoids are shown in Figure 1. Among these compounds, friedelin could be regarded as a representative triterpenoid in EBS because friedelin was determined as a dominant compound in the active fraction. Figure 2 shows the GC-

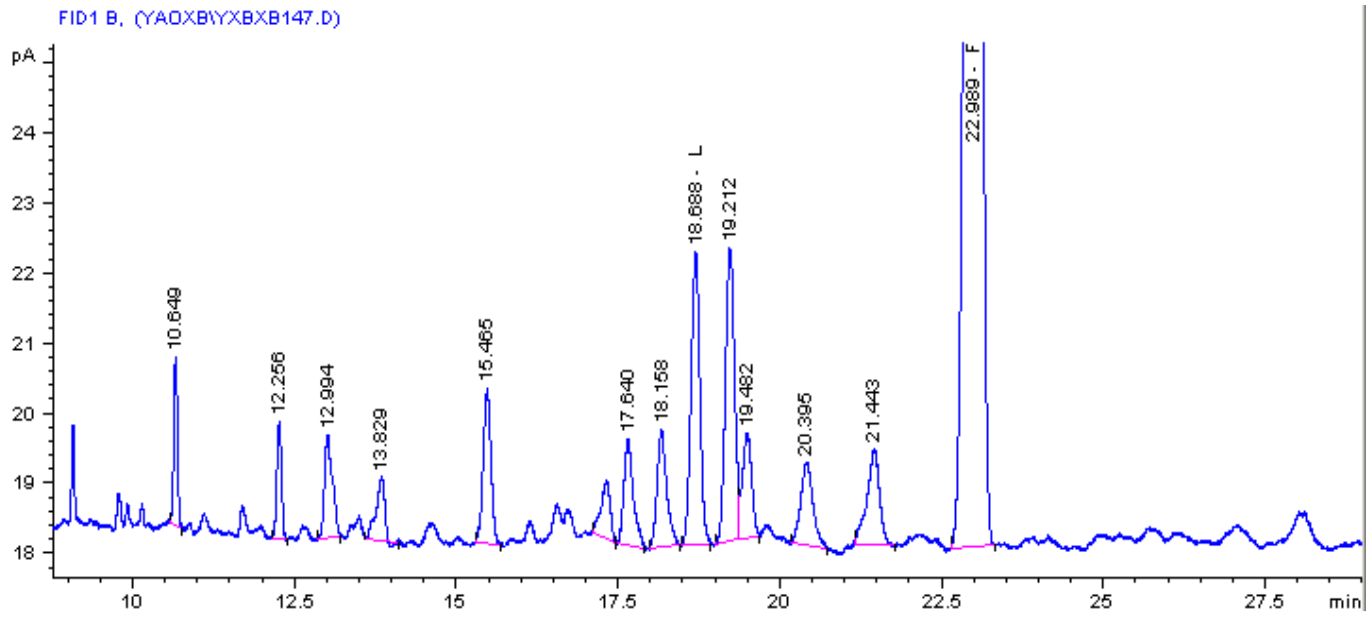

Figure 2. GC-MS chromatogram of EBS. The chromatographic conditions are given in Section 'Preparation of EBS and identification of triterpenoids'.

Table 1. Inhibitory effects on the growth of P388 mouse leukemia cells by EBS*.

Sample level	Inhibition rate (%)					Effects
Vincristin (mol/L)	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	Strong effects
	93.1	94.3	94.8	94.7	93.2	
EBS (mg/mL)	1	0.25	0.063	0.016	0.004	A little effects
	90.7	98.2	90.4	57.7	0	

MTT reducing method. Effect time: 48 h.

Table 2. Inhibitory effects on the growth of A549 human lung adenocarcinoma cells by EBS*.

Sample	Inhibition rate (%)					Effects
Vincristin (mol/L)	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	Weak effects
	71.3	71.6	66.5	44.3	9.6	
EBS (mg/mL)	1	0.25	0.063	0.016	0.004	A little effects
	70.2	90.6	24.8	0	0	

SRB protein staining method. Effect time: 72 h.

MS chromatogram describing the chemical profile of EBS.

Anti-tumor active filtration of EBS *in vitro*

The SRB protein staining and MTT reducing method were used to measure the growth inhibitory effects of P388 and A549 tumor cells, respectively. Results were shown in Tables 1 and 2. Vincristin had notable inhibitory effect on P388 cells while a little weaker effect on A549 cells. The inhibition rate of P388 was 57.7% with an EBS level of 0.016 mg/mL while the inhibition rate of A549 was 24.8% with an EBS level of 0.063 mg/mL. Further evidence suggested that EBS had shown inhibitory effect on P388 and A549 cell lines and had anti-tumor activity.

Inhibitory effects on the tumor-bearing mice S180 model by EBS

Results showed that both cyclophosphamide and EBS

Table 3. Effects on the sarcoma-loaded mice S180 model by EBS.

Group	Dosage (mg/kg)	Animal number	Tumor weight (g)	Tumor inhibition rate (%)
Control	-	10	1.81±0.60	-
CPA*	30	10	0.06±0.02**	96.68
EBS	1600	10	1.13±0.44*	37.57
EBS	800	10	1.14±0.41*	37.02
EBS	400	10	1.50±0.69	17.13

* Compared to control group, **P<0.01, *P<0.05; CPA, cyclophosphamide, single ip administration.

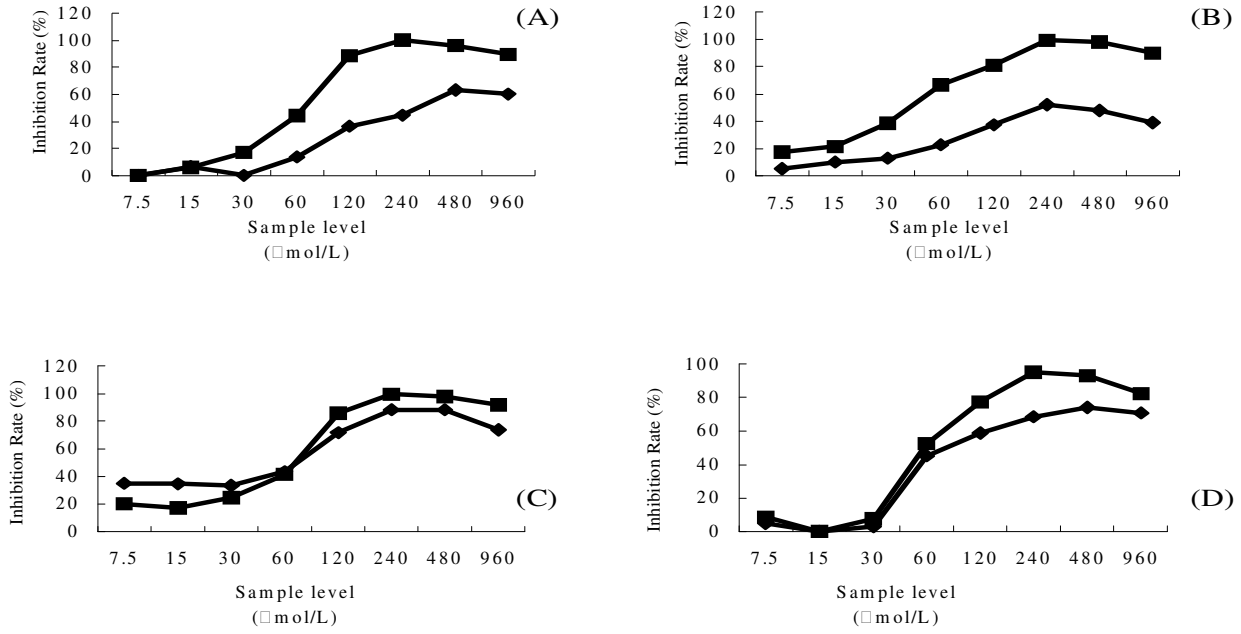

Figure 3. The inhibition rate and IC_{50} values of four tumor cells by Friedelin isolated from EBS. (A) A375 cell lines, (∅) Effect time: 24 h, (IC_{50} value: 356.54 μmol/L), (■) Effect time: 48 h, (IC_{50} value: 61.52 μmol/L); (B) L929 cell lines, (∅) Effect time: 24 h, (IC_{50} value: 665.42 μmol/L), (■) Effect time: 48 h, (IC_{50} value: 36.94 μmol/L); (C) Hela cell lines, (∅) Effect time: 24 h, (IC_{50} value: 61.25 μmol/L), (■) Effect time: 48 h, (IC_{50} value: 64.69 μmol/L); (D) THP-1 cell lines, (∅) Effect time: 24 h, (IC_{50} value: 85.10 μmol/L), (■) Effect time: 48 h, (IC_{50} value: 58.04 μmol/L).

had notable inhibitory effects on the sarcoma-loaded mice S180 model, which led to a depressed trend of tumor weights. The tumor inhibition rate of cyclophosphamide, EBS high dose, intermediate dose and low dose groups was 96.68, 37.57, 37.02 and 17.13%, respectively. In addition, it was notable for 30 mg/kg dose of cyclophosphamide and 1.6 g/kg dose, 0.8 g/kg dose of EBS to have statistical significance on inhibitory effects of tumor weights with Student's *t* test method compared to the control group ($P < 0.05$ or $P < 0.01$, Table 3).

Anti-tumor test of friedelin isolated from EBS *in vitro*

Figure 3 showed the inhibition rate of four tumor cells and the IC_{50} value by friedelin sample. From these data, it was proved that friedelin had notable inhibitory effect on Hela and THP-1 cells at 24 h. The inhibition rate of two cells had risen to 50% when the sample level was 60 μmol/L, which suggested that friedelin had higher sensitivity on these cells. On the other hand, friedelin had a little weaker inhibitory effect on A_{375} and L_{929} cells. It did not have notable effect until the effect time was up to 48 h. The inhibition rate of two cells had risen to 50% when the sample level was added to 240 μmol/L and the effect time was 24 h, which suggested that friedelin had weaker sensitivity on these two cells.

Figure 4 showed the inhibition rate of three adherent tumor cells compared to positive control, de-methyl-cantharidin. It could be demonstrated that friedelin and its positive control had similar inhibitory ability of A375, L929 and Hela adherent cells when the effect time was 24 h, while friedelin had stronger inhibitory effect on these three cells than de-methyl-cantharidin when the effect

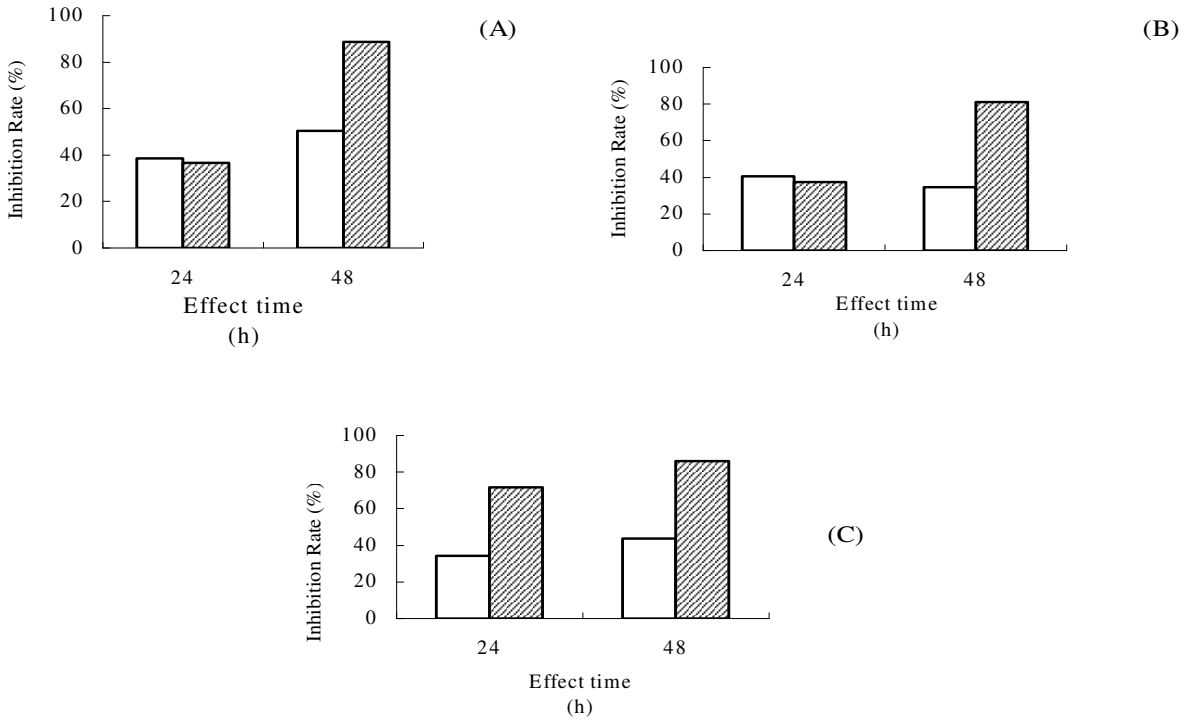

Figure 4. The inhibition rate of three adherent tumor cells by friedelin compared to de-methyl- cantharidin. (A) A375 cell, (B) L929 cell and (C) Hela cell. The concentrations of both samples were 120 μmol/L.

time was 48 h, which suggested that friedelin displayed a rather strong anti-tumor activity.

DISCUSSION

Bamboo shavings, which can be used as traditional Chinese medicinal materials, has mainly been applied for the field of Chinese medicine so far while it has not good application in other countries. However, the development of its extraction process and biological functions has not been focused on yet.

Some previous studies demonstrated the primary anti-tumor activities of bamboo leaf (Seki et al., 2008; Kim et al., 2007) or bamboo grass extracts (Tsunoda et al., 1998; Nagasawa et al., 2000), which have a bit important significance for our study. For instance, the extract of bamboo leaves could significantly inhibit tumor growth in S-180 and C38 tumor models and significantly prolonged overall survival of rats (Seki et al., 2008). The acetone fraction of bamboo leaf could enhanced leukemia cell differentiation (Kim et al., 2007).The extract of bamboo grass leaves could be a promising agent for the protection and therapy of breast and other types of tumors (Tsunoda et al., 1998). Research data in the present study primarily showed the anti-tumor activity of EBS. Therefore, it can be accepted that there may have some anti-tumor components which are widely distributed in different parts of bamboos. However, it is still uncertain whether the active compounds exerting the anti-tumor activity are similar with each other in bamboo leaves, bamboo grass or bamboo shavings.

Study on the anti-tumor effect of triterpenoids, which has become mainly biological active components in bamboo shavings, is widely concerned. Researches reported by Akinisa et al. (2001) suggested that 38 of multiflorane-type triterpenoid derivatives had been evaluated for their inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by the tumor promoter 12-O-tetradecanoylphorbol-13- acetate in Raji cells as a primary screening test for anti-tumor promoters. On the other hand, our study about anti-tumor activity of EBS and its monomer showed that friedelin monomer isolated from EBS had inhibitory effects on the proliferation of four tumor cells, A375, L929, Hela and THP-1 to a different extent, with a time-dose resistant relationship. Furthermore, friedelin had notable inhibitory effects on Hela and THP-1 cells at 24 h, which suggested that friedelin had higher sensitivity on these cells, but it had weaker sensitivity on A375 and L929 cells. Therefore, EBS had notable inhibitory effects on the sarcoma-loaded mice S180 model, which led to a depressed trend of tumor weights. It was notable for 1.6 and 0.8 g/kg dose of EBS to have statistical significance on inhibitory effects of tumor weights with Student's *t*-test method compared to the control group.

Recently, many triterpenoids isolated from natural plants have anti-tumor effects. Li et al. (2000) studied triterpenes

of betula platyphylla (TBP) which showed potent anti-tumor effects on melanoma B_{16} and sarcoma S_{180} and demonstrated that inducing apoptosis and arresting tumor cell in G_0/G_1 phase was one of anti-tumor mechanism of TBP. The triterpenoid fraction from the rhizomes of *Astilbe chinensis* could not only significantly inhibit the growth of mice transplantable tumor, but also remarkably increase splenocytes proliferation, NK cells activity and the level of IL-2 secreted by splenocytes in tumor-bearing mice, promote the DTH reaction and enhance anti-SRBC antibody level in naive mice (Tu et al., 2008). Furthermore, triterpenoids isolated from natural plants could decrease tumor cell proliferation and induce apoptosis (Mujoo et al., 2001; Ikeda et al., 2003) and the similar effect of some novel triterpenoids had been reported (Suh et al., 1999; Lapillonne et al., 2003) just as triterpenoids isolated from bamboo shavings in the present study.

Pentacyclic triterpenes from *Chrysobalanceae* species had notable inhibitory effects on P388 leukemia cell lines (Fernandes et al., 2003). Our results in this study suggested that EBS extracted from bamboo had good anti-tumor effects. On one hand, the inhibition rate of P388 was 57.7% with an EBS level of 0.016 mg/mL. Therefore, it had notable inhibitory effects. On the other hand, friedelin displayed a rather strong comparing with its positive control, de-methyl-cantharidin. Compared with other chemical medicines, the EBS and friedelin have lower anti-tumor activity, but the safety of EBS had been evaluated, it was confirmed that EBS is safety and has no side-effect (Zhang et al., 2004). Therefore, this extract of bamboo shavings had a great potential to be developed as anti-tumor health foods and natural medicine. Therefore, it can be concluded that friedelin can be regarded as one of the most important active factors in EBS though it is infrequent to report the physiological function of friedelin. But it is still unknown whether other terpenoids in EBS have anti-tumor effects and their corresponding mechanism. Overall, it can be predicted that EBS has a great potential to be applied in corresponding fields for its anti-tumor activity.

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Full Length Research Paper

Preventive and therapeutic effects of antler collagen on osteoporosis in ovariectomized rats

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The present study was aimed at evaluating the preventive and therapeutic effects of collagen extracted from Sika deer velvet on osteoporosis in ovariectomized rats. Histomorphometric indices, serum biochemical parameters and biomechanical properties were measured in ovariectomized rats treated with/without antler collagen and in sham-operated rats. Compared with the ovariectomized group, significant elevation in the levels of bone mineral density (BMD), nitrogen monoxide (NO), NO synthase, bone-Ca, bone-P, bone morphogenetic protein (BMP), trabecular bone volume (TBV), number of trabecular (N), mean trabecular plate thickness (MTPT), and biomechanical properties, while reduction in the level of serum alkaline phosphatase (ALP), and mean trabecular plate separation (MTPS) were observed in antler collagen-treated groups. The extracted collagen was found to play a role in the prevention and treatment of osteoporosis in ovariectomized rats.

Key words: Antler, collagen, osteoporosis, preventive, therapeutic.

INTRODUCTION

Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to bone fracture. Reduction in the secretion of sex hormones is one of the important causes of osteoporosis. Hormone replacement therapy and bone resorption inhibitors such as selective estrogen receptor modulators, bisphosphonates, and calcitonin are widely used in the treatment and prophylaxis of post-menopausal osteoporosis. Sika deer velvet, an efficient traditional medicine for strengthening bones and tendons, has been used for thousands of years in China, but its substance foundations for use in curing diseases has not yet been elucidated. Sika deer velvet has a complex chemical composition (Kim, et al., 1999; Wang, et al., 2003; Feng, et al., 1997; Hemmings and Song, 2004; Banks and Newbrey, 1983; Chen, et al., 2004), such as amino, protein, vitamin, lipid, mucoitin, microelement, mineral and etc (Wang, 2006); crude protein accounts for more than 50% of the dry weight of plum velvet, and collagen is the major component of the crude protein. Collagen occurs ubiquitously in mammals in organs such as the skin, bone, joint cartilage, blood vessels, tendons, and even teeth. Due to factors such as low immunogenicity, absorption, and cell growth promotion ability, collagen has been widely used in the pharma-ceutical industry and medical research and has many clinical applications (Kawahara et al., 2007; Li et al., 2007; Ponsioen et al., 2008).

However, it has been approved that collagen gotten from different materials different in many aspects (Jiang, 2006). To investigate the chemical basis for the efficacy of collagen extracted from Sika deer velvet to strengthen bones and tendons, we evaluated the preventive and therapeutic effects of antler collagen on osteoporosis.

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Abbreviations: BMD, Bone mineral density; **NOS,** nitric monoxide synthase; **BMP,** bone morphogenetic protein; **TBV,** trabecular bone volume; **N,** number of trabecular; **MTPT,** mean trabecular plate thickness; **ALP,** alkaline phosphatase; **MTPS,** mean trabecular plate separation; **SHAM,** sham-operated group; **OVX,** ovariectomy group; **OVX + N,** ovariectomy with nilestriol treatment group; **OVX + L,** ovariectomy with low-dose antler collagen treatment group; **b-Ca,** bone calcium; **b-P,** bone phosphorous; **H and E,** hematoxylin and eosin stain.

MATERIALS AND METHODS

Chemicals

Nilestriol was purchased from Shanghai medicine group; pento

barbital sodium was obtained from Shanghai chemical reagent factory; bone morphogenetic protein (BMP) radioimmunoassay (RIA) kit, nitric monoxide synthase (NOS) kit, and Ca kit were obtained from Nanjing Jiancheng biological engineering research institute. All the chemicals were of analytical grade and used without further purification.

Instruments

GF-D800 Semi-automatic biochemical analyzer (Caihong Company, ShangDong), UVmini-1240 spectrophotometer (Japan), EG 1150H tissue embed machine (LEICA Germany), and dual-energy X-ray absorptiometer (DEXA, Italy) were used in the present study.

Preparation of collagen

After removing the skin and flesh, the antlers of fresh Sika deer velvet (500 g) provided by Dongfong medicine pharmaceutical factory (Jilin, China) were cut into 1-cm^3 pieces and washed with distilled water at 4℃; subsequently, the pieces were homogenized. The homogenate was incubated in 1000 ml of distilled water for 48 h at 60℃ and then centrifuged. The supernatant was dialyzed against distilled water for 24 h (molecular weight cut-off of 8 ~ 15 kDa). The dialysate was lyophilized and stored at -20℃ until further use.

Rat osteoporosis model

All the rats were anesthetized using 0.4% pentobarbital sodium at doses of 50 mg/kg body weight. Bilateral ovariectomies were performed in the rats via a dorsal approach (Xu et al., 2006). Sham operation was performed using the same procedure, but without removing the ovaries.

Experimental design

Fifty female wistar rats (weight, 220 ± 10 g; 3 months) obtained from the Changchun Gaoxin experimental animal center (Changchun, China) were randomly divided into the following 5 groups (n = 10, for each group): sham-operated group (SHAM), ovariectomy group (OVX), ovariectomy with nilestriol treatment group (OVX + N), ovariectomy with high-dose antler collagen treatment group (OVX + H), and ovariectomy with low-dose antler collagen treatment group (OVX + L). After 15 days of surgery, the OVX + N group was orally administered nilestriol at a dose of 0.36 $mg \cdot kg^{-1}$ every 2 weeks, and the OVX + H and OVX + L groups were orally administered antler collagen at doses of 0.5 $g \cdot kg^{-1} \cdot d^{-1}$ and 0.1 $g \cdot kg^{-1} \cdot d^{-1}$, respectively. The SHAM and the OVX groups received normal saline (0.9%) orally at the same volume. The treatment was continued for 90 days.

Preparation of the samples

Blood samples were collected from the abdominal aorta of the experimental rats, and the serum was immediately separated by centrifugation (4℃) at 1,500 g for 15 min and stored at -20℃. The right femurs and the third lumbar vertebrae were resected, the adhering connective tissue, cartilage, spinous processes, and articular processes were removed, and the bones were vacuum dried for 24 h. The third lumbar vertebrae were decalcified 3 times using trichloroacetic acid (24 h each time), and the decalcifying fluid was pooled for further use.

Measurement indicators

Bone mineral density (BMD) was determined by dual-energy X-ray absorptiometer. The levels of bone calcium (b-Ca), bone phosphorous (b-P) serum alkaline phosphatase (ALP), BMP, nitrogen monoxide (NO) and NO synthase were detected by using the corresponding kits. The mean trabecular plate thickness (MTPT, μm), mean trabecular plate separation (MTPS, μm), trabecular bone volume (TBV, %), and other histomorphometric parameters ratio were measured by using the BI-2000 system after the rats have been sacrificed, left femur taken, fixed in 10% formalin, decalcified in 10% formic acid, dehydrated using graded ethanol, vitrified by dimethylbenzene, paraffin section, hematoxylin and eosin (H and E) stained, at 100 times, under the metaphyseal cortical growth plate near 1 - 4 mm, to obtain vision range measurements. The left femur was used in calculating the bone weight coefficient and bone biomechanical testing, the maximum load, maximum deformation, bone stress and strain. All the statistical tests were performed using Microsoft Excel. Statistical significance of the difference between the groups was tested by unpaired Student's *t* tests. $P < 0.05$ was considered as statistically significant.

RESULTS

Amino acid analysis

As shown in Table 1, glycine (32.24%) was the major component in the amino acid hydrolysates of the extracted collagen. Proline and alanine accounted for 12.64 and 10.78% of the extracted collagen, respectively. The levels of histidine and tyrosine were low, that is, 0.57 and 0.21%, respectively. Cystine was not detected; hydroxyproline was also a component of the extracted hydrolysate, and the ratio of hydroxyproline to proline was 0.8, which was almost similar to that of type I collagen (0.7 ~ 0.8) (Jiang, 2006).

BMD and b-Ca

As shown in Table 2, compared to the SHAM group, the BMD value and the bone-Ca (b-Ca) level were lower ($P < 0.001$), and the b-P was lower ($P < 0.05$) in the OVX group. Compared to the OVX group, the BMD values of both the antler collagen-treated groups ($P < 0.01$ or $P < 0.05$) and the OVX + N group were higher ($P < 0.001$). The b-Ca was higher($P < 0.05$) than that of the OVX group in the OVX + H , OVX+L , and OVX + N groups .The b-P level was higher ($P < 0.05$) than that of the OVX group in the OVX + H , and OVX + N groups .

Biochemical markers

Table 3 shows that the levels of BMP, NO, and NO synthase were lower ($P < 0.01$), but the level of ALP was higher ($P < 0.05$) in the OVX group than in the SHAM group. Compared to the OVX group, the levels of NO and NO synthase were higher and that of ALP were lower in both the collagen-treated groups and the OVX + N group.

Table 1. Results of amino acid analyze of velvet antler collagen from sika deer.

Amino acid	Content (%)	Amino acid	Content (%)
Gly	32.24	Leu	2.59
Pro	12.64	Thr	2.33
Ala	10.78	Val	2.22
q-Pro	10.20	Phe	1.22
Glu	8.46	Ile	1.08
Asp	4.55	His	0.57
Arg	4.42	Tyr	0.21
Ser	3.56	Met	—
Lys	2.94	Cys	—

Table 2. BMD, b-Ca and b-P levels.

Target Group	g/kg	n	BMD (g/cm^2)	b-Ca (mmol/L)	b-P (mmol/L)
SHAM	—	8	0.185 ± 0.044	9.5 ± 1.1	62 ± 15
OVX	—	8	0.063 ± 0.014$^{\Delta\Delta\Delta}$	7.7 ± 0.4$^{\Delta\Delta\Delta}$	82 ± 14$^{\Delta}$
OVX + N	0.00036	8	0.127 ± 0.013***	8.7 ± 1.7*	80 ± 16*
OVX + H	0.5	8	0.084 ± 0.010**	8.9 ± 1.2*	79 ± 14*
OVX + L	0.1	8	0.080 ± 0.017*	8.8 ± 1.4*	72 ± 15

Values have been indicated as mean ± standard deviation; *P < 0.05 versus OVX ; **P < 0.01 versus OVX ; ***P < 0.001 versus OVX; Δ P < 0.05 versus SHAM; ΔΔΔ P < 0.001 versus SHAM.

Table 3. Biochemical parameters.

Group	g/kg	n	ALP	BMP (ng/mL)	NO (μmol/L)	NO synthase (μg/mL)
SHAM	—	8	200 ± 42	2.2± 0.32	54± 0.88**	58 ± 3.9**
OVX	—	8	303 ± 86$^{\Delta\Delta}$	1.6 ±0.22$^{\Delta\Delta}$	24± 4.9	38 ± 4.6
OVX + N	0.00036	8	205 ± 37*	2.1± 0.28**	30± 2.6*	41 ± 5.8*
OVX + H	0.5	8	228 ± 103*	1.9± 0.31*	39± 6.9**	49 ± 9.0**
OVX + L	0.1	8	218 ± 72*	1.7 ± 0.22	30± 3.8*	47 ± 3.3**

Values have been indicated as mean ± standard deviation; *P < 0.05 versus OVX; **P < 0.01 versus OVX; ΔΔP < 0.01 versus SHAM.

BMP level in the OVX + N group and the OVX + H group was higher than that in the OVX group. However, the BMP level in the OVX + L group did not differ significantly from that of the OVX group.

Bone tissue and morphogenetic indices

The values of histomorphometric indices in the OVX group were lower than those in the SHAM group. Compared to the OVX group, there was no marked difference in the MBCT and the MTPT in the collagen-treated groups and the OVX + N group, but MTPT in the OVX+N groups. On the other hand, MTPS, and number of trabecular values in the OVX + H, the OVX+L and OVX + N groups were higher than those in the OVX group. The TBV was higher in the OVX+N (P < 0.01) and OVX+H (P < 0.05) groups (Table 4 and Figure 1).

Biomechanical properties

The values of biomechanics indices in the OVX group were lower than those in the SHAM group. Compared to the OVX group, there was marked difference in maximum load (M-N), maximum deflection (M-D), bone stress and bone strain in the collagen-treated groups and the SHAM group (P < 0.05 or P < 0.01) (Table 5).

Table 4. Histomorphometric parameters.

Target Group	g/kg	n	MBCT (μm)	MTPS (μ)	TBV (%)	Number of bone trabecula	MTPT (μm)
SHAM	—	8	12.19±0.15*	9.5±1.0***	36.79 ± 3.2***	3.55 ±0.69***	8.33 ± 0.21**
OVX	—	8	12.00±0.20$^{\Delta}$	16.9 ±1.6$^{\Delta\Delta\Delta}$	19.43 ±4.6$^{\Delta\Delta\Delta}$	0.64 ±0.50	8.10 ±0.12$^{\Delta\Delta}$
OVX + N	0.00036	8	12.15±0.27	13.1±1.5***	26.20 ±4.5**	2.00±0.77***	8.23 ± 0.13*
OVX + H	0.5	8	12.06±0.27	14.1±2. 8**	24.82 ± 5.6*	1.69±0.63***	8.20 ± 0.16
OVX + L	0.1	8	12.01±0.28	14.3 ±3.0*	24.20 ± 7.8	1.55 ± 1.04*	8.17 ± 0.13

Values have been indicated as mean ± standard deviation; *P < 0.05 versus OVX; **P < 0.01 versus OVX; ΔP<0.05 versus SHAM; ΔΔP < 0.01 versus SHAM; ΔΔΔP < 0.001 versus SHAM.

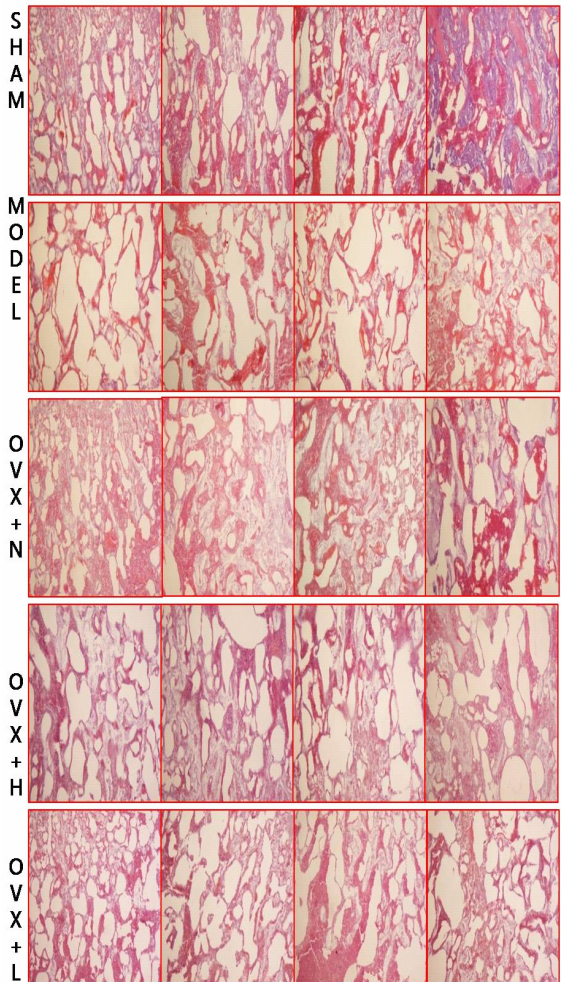

Figure 1. Effects of CSDV on static histomorphometric indexes of OP rats.

DISCUSSION

The osteoporosis model was established successfully in the present study. The values of BMD, bone-Ca, biochemical parameters, histomorphometric parameters and biomechanical properties were lowered in the OVX group than in the SHAM group. After 90 days of treatment, there was a marked increase in the BMD, bone-Ca, biochemical parameters, histomorphometric parameters and biomechanical properties in the collagen-treated groups compared with that in the OVX group. These results indicate that Sika deer velvet antlers play a role in the treatment and/or prevention of osteoporosis in OVX rats.

Human bones constituted of bone mineral and bone matrix; the ratio is about 2:1 (Jiang, 1987). The main component of bone mineral is Ca; the main component of bone matrix is collagen. Osteoporosis is mainly due to the loss of Ca and collagen degradation. As the result show, the level of BMD and the bone–Ca and P in collagen-treated rats increased (P < 0.01, P < 0.05), when the level of ALP and BMP was adjusted (P < 0.05; P < 0.01). Descriptions of these indicators show that the collagen increases the bone mineral level of OVX rats markedly. The above results show that rats treated with collagen were meliorated at corresponding indicators, and the bone matrix level in OVX rats, and collagen was well absorbed. The absorbing of collagen amends the indicators of bone biomechanics, as maximum load, maximum deflection and bone strain were higher and enhanced while brittleness was lowered in other to achieve the purpose of the prevention of osteoporosis.

The first step in the formation of new bone is collagen synthesis, which interlaced with each one another, while forming bone matrix net. Next, hydroxyl calcium phosphate deposits in the net. Hence, collagen is important for bone growth and bone development. It has been reported in literature that hydroxyproline, the characteristic collagen amino acid, was carrier absorption of calcium; it was thus concluded that hydroxyproline in collagen promote the absorption of calcium (Li et al., 2000). Through our experiments, collagen content of hydroxyproline to the total amino acid content was about 30% (Table 1), while collagen is the most abundant protein content above 50% in deer velvet. The results indicate that, deer antler and collagen may be used to prevent and treat osteoporosis and increase bone density through the use of hydroxyproline.

Furthermore, experiments have been carried out to show that collagen promote osteoblast in rat proliferation. The results showed that the collagen treated group proliferate more quikly than none treated group (Li et al.,

Table 5. Biomechanical properties.

Target Group	g/kg	n	M-N (N)	M-D (mm)	Bone-stress (N/mm^2)	Bone-strain (%)
SHAM	—	8	141.2±26.2**	0.88±0.27*	141.4±21.1**	4.60±1.24**
OVX	—	8	$105.1±23.3^{\Delta\Delta}$	$0.66±0.15^{\Delta}$	$112.7±25.2^{\Delta\Delta}$	$3.29±0.70^{\Delta\Delta}$
OVX + N	0.00036	8	128.1±21.7*	0.82±0.17*	146.9±29.1**	4.31±0.75**
OVX + H	0.5	8	127.5±26.0*	0.83±0.21*	139.4±27.1*	4.21±1.00**
OVX + L	0.1	8	122.9±17.9*	0.80±0.13*	135.5±19.1*	4.09±0.74*

Values have been indicated as mean ± standard deviation; *P < 0.05 versus OVX; **P < 0.01 versus OVX; ΔP < 0.05 versus SHAM; ΔΔP < 0.01 versus SHAM.

2009). These show that collagen act most likely through the promotion of osteoblast proliferation to prevent osteoporosis. The levels of NO and NO synthase, which are responsible for the activation of many cytokines, were also measured. The results showed that the levels of NO and NO synthase in the OVX group were significantly lower than those of the SHAM group, and those of the antler collagen-treated groups. Collagen treatment had a remarkable impact on the levels of NO and NO synthase. This suggests that the antiosteoporotic effect of antler collagen was mediated by the upregulation of NO and NO synthase that led to the stimulation of osteoblast proliferation and inhibition of osteoclast activity.

In summary, the Sika deer velvet collagen could significantly increase BMD and prevent osteoporosis induced by estrogen deficiency in ovariectomized rats. Antler collagen may thus have potential therapeutic applications in the treatment of osteoporosis.

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Full Length Research Paper

Evaluation of subchronic dietary fumonisin B_1 on nutrient digestibility and growth performance of rats

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Fumonisin B_1 (FB_1), a toxin produced by *Fusarium verticillioides* (*Fusarium moniliforme*) and other *Fusarium* species which grow on maize worldwide, has been documented to cause various physiological responses in animals. Thirty-nine female Wistar rats randomly assigned to three treatment groups were used to assess the effects of dietary FB_1 on nutrient utilization and growth performance. Each group received one of the three diets containing 0.20, 10.0 and 20.0 mg FB_1/kg constituting diets 1, 2 and 3, respectively. The animals were weighed weekly and proximate chemical compositions of the diets and the faecal samples collected from the rats on each diet were determined using standard methods. Dietary FB_1 significantly ($P < 0.05$) influenced nutrient digestibility, feed intake, feed conversion efficiency and relative weight gain. Rats fed diets 2 and 3 had relative weight gains of 87.2 and 66.2% of the rats fed diet 1, respectively. Rats on diet 1 were about 104.5 and 160.6% more efficient in feed conversion compared to those on diets 2 and 3, respectively. Dietary exposure to FB_1 at a concentration of about 10 mg/kg or higher for a period of 35 days is a potential health risk that reduced nutrient utilization by adversely affecting proper nutrient digestion, absorption and/or metabolism, resulting in poor growth rates in Wistar rats. This study revealed that adverse effects of FB_1 on nutrient digestibility and utilization play a significant contributory role in poor growth performance usually associated with animals exposed to diets containing FB_1.

Key words: Fumonisin B_1, growth performance, mycotoxin, nutrient digestibility, rats.

INTRODUCTION

Maize (*Zea mays* L.), as a main energy source, is the major cereal used in the production of livestock feeds and it is reported to be particularly vulnerable to degradation by mycotoxigenic fungi (Lillehoj, 1987; Munkvold and Carlton, 1997). *Fusarium verticillioides* (Sacc.) Nirenberg (*F. moniliforme* Sheld.) is one of the most prevalent toxigenic fungi associated with dietary staples such as maize for human and animal consumption worldwide (Nelson et al., 1991; Kedera et al., 1992). The fungus is present in virtually all maize samples (Marasas et al., 2001).

Fumonisins are mycotoxins produced principally by *F. verticillioides* (Gelderblom et al., 1988; Marasas et al., 2001). Worldwide distribution of fumonisins in maize, feeds, and foodstuffs and their implications in human and animal health has been comprehensively reviewed (Dutton, 1996; Marasas, 1993, 1996; Marasas et al., 2001). Pathogenic effects of fumonisins include fatal diseases in farm and laboratory animals such as equineleukoencepalomalacia (ELEM) (Wilson et al., 1990), porcine pulmonary oedema (PPE) (Harrison et al., 1990), hepatotoxicity, hepatocarcinogenicity (Gelderblom et al., 1991, 2001) and nephrotoxicity (Norred et al., 1996; Bucci et al., 1998). Several naturally occurring fumonisins are known; fumonisins B_1 (FB_1) has been reported to be the most abundant and most toxic which represents approximately 70% of the total concentration in naturally contaminated foods and feeds, followed by

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Abbreviations: **FB_1,** Fumonisin B_1; **ELEM,** equineleukoencepalomalacia; **PPE,** porcine pulmonary oedema; **HPLC,** high performance liquid chromatography; **DM,** dry matter; **OM,** organic matter; **CP,** crude protein; **EE,** ether extract; **CF,** crude fibre; **NFE,** nitrogen-free extract; **FCR,** feed conversion ratio; **TER,** transepithelial resistance.

Table 1. Gross composition (%) of the experimental diets.

Ingredient	Diet 1	Diet 2	Diet 3
Non-inoculated maize	60.00	56.00	52.00
Inoculated maize*	-	4.00	8.00
Soybean meal	20.00	20.00	20.00
Wheat offal	12.25	12.25	12.25
Fish meal	5.00	5.00	5.00
Dicalcium phosphate	1.25	1.25	1.25
Vegetable oil	0.50	0.50	0.50
Oyster shell	0.25	0.25	0.25
Minerals/vitamins premix**	0.20	0.20	0.20
Salt	2.50	2.50	2.50
Calculated nutrients			
Crude Fibre (%)	5.23	5.22	5.20
Crude Protein (%)	20.08	20.03	20.01
DE*** (kcal/kg)	2972.48	2972.48	2972.48

*Infected with *Fusarium verticillioides* inoculums; **To provide per kg diet: Vitamin A (10,000 i.u.), vitamin D (20,000 i.u.), vitamin E (5 i.u.), vitamin K (2.5 mg), choline (350 mg), folic acid (1 mg), manganese (56 mg), iodine (1 mg), iron (20 mg), copper (10 mg), zinc (50 mg) and cobalt (1.25 mg); ***calculated values.

fumonisins B_2 (FB_2) and B_3 (Murphy et al., 1993; Norred, 1993). Consequently, toxicological studies on the fumonisins have been concentrated on FB_1 (Gbore, 2009).

The mode of action of fumonisins is primarily explained by interference with the *de novo* synthesis of complex glyco-sphingolipids (Wang et al., 1991) which results in disturbances of cellular processes such as cell growth, cell differentiation and cell morphology, endothelial cell permeability and apoptosis (EC, 2000). Significant adverse effects of FB_1 on food consumption, body weights and body weight gains in animals, especially rats, have been well documented (Bondy et al., 1998; Voss et al., 1998; Gelderblom et al., 1988, 1994; NTP, 2001; Swamy et al., 2002; Theumer et al., 2002). However, the impact of FB_1 on nutrient digestibility and its role in the observed depressed feed consumption, body weights and body weight gains in rats exposed to FB_1 have not been reported. This study was designed because fumonisins are known to be consumed by farm animals and is the causative agents or suspected contributing factor in farm animals' diseases and poor growth performances. The objective of this study was to assess the effects of dietary exposure to FB_1 on nutrient digestibility by rats and its contributory role in poor growth performance which is usually associated with exposure to fumonisins.

MATERIALS AND METHODS

Experimental site and animals

Thirty-nine mature female Wistar rats (*Rattus norvegicus*) obtained from a commercial breeder in Benin City, Edo State, Nigeria, were housed in wire mesh rat cages at the Animal House of the Department of Biochemistry, Adekunle Ajasin University, Akungba Akoko, Nigeria, where the feeding trial was carried out between May and July, 2009. The study was approved by the Institutional Committee on the care and use of laboratory animals.

FB_1 production and experimental diets

Maize grits in 500 g quantities were placed into autoclavable polypropylene bags and soaked with 200 ml of distilled water for 2 h, then autoclaved for 1 h at 121°C and 120 kPa. The autoclaved maize grits were then cultured with a toxigenic strain of *F. verticillioides* (MRC 286) obtained from the Plant Pathology Laboratory of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria to produce FB_1 as described previously (Nelson et al., 1994). Uncultured maize grits and the cultured maize grits were used to formulate three diets. In addition to FB_1, dietary contents of aflatoxin and other common *Fusarium* mycotoxins including deoxynivalenol (DON, vomitoxin), T-2 toxin and zearalenone were also checked by using mycotoxin quantitative CD-ELISA test kits (Neogen, Lansing, MI, USA) and reconfirmed by using High Performance Liquid Chromatography (HPLC) analyses as described by Shephard et al. (1990). All other common mycotoxins screened were found to be negligible. The concentrations of FB_1 in the diets were adjusted to 0.2, 10.0 and 20.0 mg/kg constituting diets 1 (control diet), 2 and 3, respectively.

Experimental model

After 3 weeks of physiological adjustment period, the rats were allocated by weight to each of the three diets (n = 13 rats per treatment) such that the initial body weights of the rats were uniform across the dietary groups. The gross compositions of the pelleted diets are shown in Table 1. The rats were provided with fresh clean water and appropriately weighed feed daily, and the weights of feed portions given and left uneaten after 24 h were determined. The body weight was determined weekly on a weighing scale (Ohaus Corp., Pine Brook, NJ, USA) with a precision of 0.05 g. The body weight gain of each rat was determined weekly as the weight difference in comparison to the weight in the previous week.

Table 2. Apparent nutrient digestibility (%) of Wistar rats fed with varied levels of dietary fumonisin B_1.

Nutrient	Diet 1	Diet 2	Diet 3
Dry matter	50.00 ± 0.28[a]	41.93 ± 0.23[ab]	40.95 ± 0.35[b]
Crude protein	53.29 ± 0.30[a]	48.08 ± 0.40[a]	35.96 ± 0.20[b]
Crude fibre	77.43 ±0.44[a]	72.92 ± 0.62[a]	55.11 ± 0.30[b]
Ether extract	51.92 ± 0.30[a]	48.26 ± 0.53[b]	32.13 ± 0.18[c]
Ash	63.44 ± 0.36	57.76 ± 0.31	55.96 ± 0.47
Nitrogen-free extract	98.41 ± 0.08[a]	97.68 ± 0.13[b]	97.69 ± 0.20[b]

[abc]: Means on same row with different superscripts differ significantly ($P < 0.05$).

During the last seven days of the experimental period, faecal droppings from nine rats on each diet were collected, weighed, mixed and aliquots taken daily. The daily aliquots and the respective feed samples for each animal were dried in an air-circulatory oven at 105°C for 24 h (to determine their moisture contents) and stored for further analysis.

The chemical compositions of the experimental diets and faecal samples collected, which were used to calculate the apparent digestibility of dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE), crude fibre (CF), ash and nitrogen-free extract (NFE), were determined by the methods of AOAC (1995).

Statistical evaluation

Data from this study were analyzed by one-way analysis of variance procedure of SAS (1999). The treatment means were compared using the Duncan procedure of the same software and results giving $P < 0.05$ were considered significantly different.

RESULTS

The apparent nutrient digestibility of Wistar rats fed with different concentrations of dietary FB_1 (Table 2) showed that the apparent digestibility values for rats on diet 1 were generally higher than those on diets 2 and 3 for each variable. The CP and CF digestibility values of rats on diets 1 and 2 were significantly ($P < 0.05$) higher than the nutrient digestibility values of those on diet 3, containing the highest FB_1 concentration. The DM and NFE digestibility values of the rats on diet 3 were significantly ($P < 0.05$) lower than those on diet 1, which were however not statistically ($P > 0.05$) different from those on diet 2. The significantly lower EE digestibility values obtained for rats on diets 2 and 3 were about 7.1 and 38.1% lower than the digestibility value of EE of those on diet 1, respectively. The apparent ash digestibility values, though not significantly different across the treatments ($P < 0.05$), appeared to decline with increased dietary FB_1.

The performance variables of rats exposed to different concentrations of dietary FB_1 are shown in Table 3. The final live weights, relative weight gains (expressed as percent of initial live weights), daily feed intake and the feed conversion ratio (FCR) were significantly ($P < 0.05$) influenced by the dietary FB_1 concentrations. The daily weight gain was, however, not significantly ($P > 0.05$) influenced by the dietary FB_1 but seemed to decline with increase in dietary FB_1 concentrations. The rats fed diets 2 and 3 had relative weight gains of about 87.2 and 66.2% of the rats fed diet 1, respectively. The mean daily feed intake of rats on diet 3 was significantly ($P < 0.05$) higher than the daily feed intake of those on diet 2, which was not different from those on diet 1. Rats fed diet 1 were about 104.5 and 160.6% more efficient in feed conversion compared to those on diets 2 and 3, respectively.

DISCUSSION

The significant decline in digestibility values for all nutrients, apart from ash, with increased dietary FB_1 by the Wistar rats in this study suggested altered normal digestive and nutrient absorptive functions of the epithetlial lining of the gastrointestinal tract. Adverse influences of dietary fumonisin on normal epithelial morphology were observed by Yoo et al. (1992) and Merrill et al. (1993). Similarly, progressive erosion of the epithelial lining of the small intestine resulting from chronic exposure to *F. verticillioides* culture material containing 1.69 - 1.90 mg fumonisin/kg was observed in rabbits by Ewuola et al. (2003), while Gbore (2007) observed progressive erosion of the intestinal mucosa in experimental pigs fed with increased concentrations of dietary FB_1. Mahfoud et al. (2002) reported that the mycotoxin patulin altered the barrier function of the intestinal epithelium by inducing a rapid and dramatic decrease of transepithelial resistance (TER) in two distinct human intestinal cell lines, which have been widely used as *in vitro* models for the human intestinal epithelium in transport and toxicity studies by Maresca et al. (2001). Mahfoud et al. (2002) reported that unrelated mycotoxins can induce a similar deleterious effect on the intestinal barrier function as demonstrated by Maresca et al. (2001) using ochratoxin A. All these observations are an indication of the role mycotoxins, including FB_1, can play in non-specific gastrointestinal tract hypofunction in animals. These observations might have been responsible for the decline digestibility values of the nutrients with increased dietary FB_1. The inability of the rats fed diets 2 and 3 to efficiently utilize the essential nutrients in their feeds might have

Table 3. Performance of female Wistar rats exposed to different levels of dietary fumonisin B_1.

Variable	Diet 1	Diet 2	Diet 3
Initial weight (g)	168.93 ± 1.41	168.83 ± 1.51	169.87 ± 1.32
Final weight (g)	228.09 ± 1.10^{a}	220.43 ± 1.76^{b}	209.05 ± 1.50^{b}
Relative weight gain* (%)	35.02 ± 0.93^{a}	30.56 ± 1.16^{b}	23.06 ± 1.09^{c}
Daily weight gain (g)	1.69 ± 0.08	1.47 ± 0.07	1.12 ± 0.08
Daily feed intake (g)	17.64 ± 0.31^{ab}	16.03 ± 0.06^{b}	18.77 ± 0.68^{a}
FCR** (g/g)	10.43 ± 1.86^{b}	10.90 ±0.86^{b}	16.75 ± 0.85^{a}

*Expressed as percent of initial live weight; **feed conversion ratio; ^{abc}means on same row with different superscripts differ significantly ($P < 0.05$).

accounted for the observed significantly lower relative weight gains for these rats in this study.

In this study, the final live weights and relative weight gains of the rats declined significantly ($P < 0.05$) with increase in the dietary FB_1 after the 35-day feeding experiment. The general decline in the weight gain is an indication of the role that FB_1 could play in animal nutrition and subsequent weight gain. The lower relative change in live weights of rats fed diets 2 and 3 compared to controls are in agreement with results from similar studies that dietary FB_1 depressed feed consumption and live weight gain in rats (Bondy et al., 1998; Voss et al., 1998; Gelderblom et al., 1988, 1994; NTP, 2001; Swamy et al., 2002; Theumer et al., 2002) and lowered feed conversion efficiency in animals (Gbore, 2009).

Drastic alterations in serum protein values are often observed, as reported by Coles (1986), in association with either kidney and liver diseases or gastrointestinal diseases involving interference with protein digestion and absorption. However, Bauer et al. (1974) reported that a low albumin level might be due to increased loss of albumin in the urine, decreased formation in the liver or insufficient protein intake. The significantly lower protein digestibility values by rats on diets 2 and 3 containing *Fusarium*-inoculated grains might be an indication of the role FB1 could play in serum protein alterations as earlier reported in animals (Ewuola and Egbunike, 2008; Gbore and Egbunike, 2009) and fish (Gbore and Akele, 2010) exposed to dietary fumonisin. Serum protein and albumin syntheses have been reported to be related to the amount of available protein in the diet (Iyayi and Tewe, 1998).

The nutritive state of the animal may be dependent not only on the proper and adequate intake of protein building materials in the diet but may also be a reflection of the nutritive state existing within the animal body, reflecting alterations in metabolism. Reduced serum protein profiles and tissue protein synthesis often observed in animals exposed to *Fusarium* mycotoxins (Dänicke et al., 2006; Ewuola and Egbunike, 2008; Gbore and Egbunike, 2009) might be a reflection of altered dietary protein metabolism, including digestibility and subsequent absorption of the nutrients in the intestine of the animals as well as biosynthesis in the body systems in animals. Patulin, a secondary metabolite of a number of fungal species, has also been reported to interfere with protein biosynthesis (Arafat and Musa, 1995). The mycotoxin was found to inhibit several key biosynthetic enzymes including RNA polymerase and aminoacyl-tRNA synthetases (Arafat et al., 1985). In a study on channel catfish fed FB_1, Lumlertdacha et al. (1995) reported that osmium staining indicated that the vacuoles in the hepatocytes of the catfish contained lipid, which was suggestive of impaired lipid metabolism. The results of nutrient digestibility in this study revealed that besides altering the digestive and absorptive functions of the intestinal epithelium, dietary FB_1 can as well perturb protein and lipid metabolism in animal systems.

The significantly reduced feed efficiency of rats fed diet 3 compared to those on diet 1 despite the higher feed intake by rats on diet 3, could be attributed to immunological response by the animals as a result of the dietary FB_1. This finding corroborates the study of Gbore and Egbunike (2007) that reported reduced feed utilization in growing pigs fed diets containing ≥5 mg FB_1/kg. The finding suggests that FB_1 could have adverse effects on feed intake and nutrient utilization and the resultant weight gain in animals as earlier reported (Theumer et al., 2002; Gbore and Egbunike, 2007; Gbore, 2009), which were probably due to adverse effects of FB_1 on intestinal function in nutrient digestibility and absorption in rats.

Diets containing ≥10.0 mg FB_1/kg, generally reduced nutrient utilization by adversely affecting proper nutrient digestion, absorption or metabolism and subsequent growth performance in Wistar rats. Further studies are however warranted to clarify how dietary FB_1 contributes to lowered serum proteins profiles commonly observed in exposed animals by altering dietary nutrient digestion and absorption from the intestinal tract or interferes with protein and lipid metabolism in animal systems.

Conclusion

Fumonisin B_1 is a potent inhibitor of ceramide synthase, an enzyme critical to the metabolism of sphingolipids (Wang et al., 1991; Merrill et al., 1993). A multitude of biological activities and cellular functions has also been reported for sphingolipids (Wang et al., 1992; Merrill et

al., 1996). It is reasonable, therefore, to assume that many of the physiological effects of FB_1 are related to disruption of these pathways. However, this study revealed that dietary FB_1 concentrations of ≥10.0 mg/kg had adverse effects on nutrient utilization and subsequently contributed to reduced growth performance in the Wistar rats. The implication of this is that the adverse effects of dietary FB_1 on nutrient digestibility and utilization also play a significant contributory role in poor growth performance usually observed in animals exposed to FB_1.

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