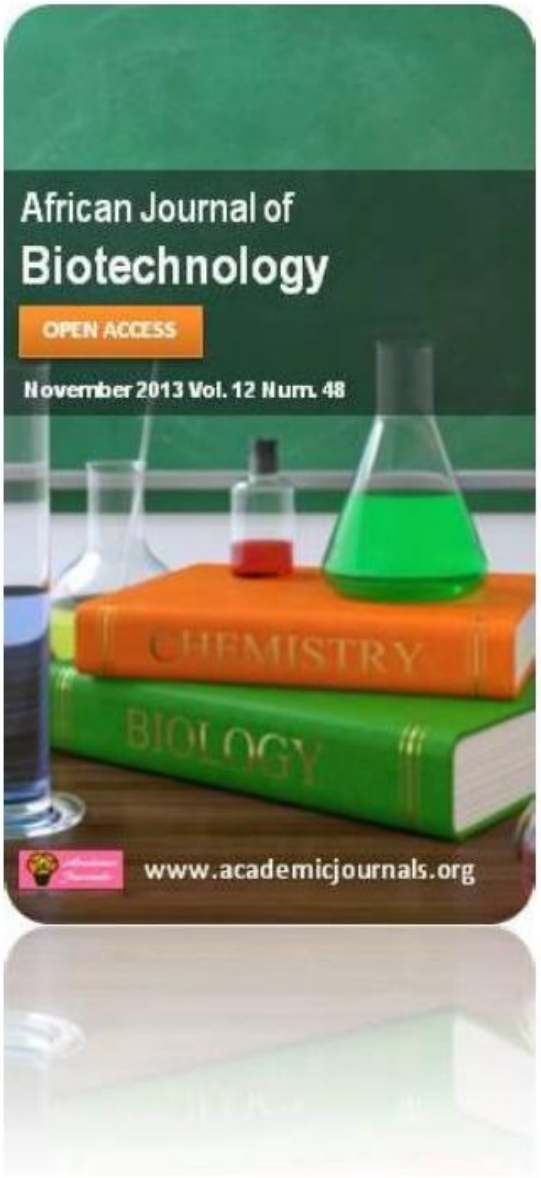

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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African Journal of Biotechnology

Review

Transgenesis: An efficient tool in mulberry breeding

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Genetic engineering is the most potent biotechnological approach dealing with transfer of specially constructed gene assemblies through various transformation techniques. Tools of recombinant DNA technology facilitated development of transgenic plants. The plants obtained through genetic engineering contain a gene or genes usually from an unrelated organisms, and are known as transgenic plants. The combined use of recombinant DNA technology, gene transfer methods and tissue culture techniques has led to the efficient transformation and production of transgenics in a wide variety of crop plants. In fact transgenesis has emerged as a novel tool for carrying out "single gene breeding" or transgenic breeding of crop plants. Identification, isolation and cloning of resistant genes is the prerequisite for development of transgenic plants for disease resistance. Identification of resistance genes on the basis of amino acid sequence, conservation enables plant breeder to monitor resistance gene segregation using appropriate DNA probe intend of testing progeny for disease resistance and susceptibility. Significant developments in plant genetic modification have been achieved in the last 15 years. Some of the success include herbicide tolerant corn, cotton, soyabeen and papaya; virus resistant corn, potato, cotton among others. In mulberry, little work has been carried out at Delhi University (south campus). They have developed drought and salinity tolerent transgenic mulberry through *Agrobacterium* mediated transformation. The overexpression of HVA1 gene from barley generates tolerence to salinity and water stress in transgenic mulberry (*Morus indica*).

Key words: Transgenic plant, mulberry, resistance, salinity.

INTRODUCTION

Genetic engineering is the most potent biotechnological approach and deals with transfer of specially constructed gene assemblies through various transformation techniques. Tools of recombinant DNA technology facilitated development of transgenic plants. Transgenic plants carry additional, stably integrated and expressed, foreign genes from trans-species. The whole process involving introduction, integration and expression of foreign genes in the host is called genetic transformation or transgenoesis. The combined use of recombinant DNA technology, gene transfer methods and tissue culture techniques has led to the efficient

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Abbreviations: CaMv, Cauliflower mosaic virus; **TGMv,** tomato golden mosaic virus; **DHFR,** dihydrofolate reductive; **PEG,** polyethylene glycol; **PAT,** phosphinothricin acetyl transferase; **PDR,** pathogen derived resistance; **CMV,** cucumber mosaic virus; **AOS,** active oxygen species; cP-MR, coat protein mediated resistance; **UPLC,** ultra-performance liquid chromatography; GUS, β-gluconidas.

transformation and production of transgenic in a wide variety of crop plants.

TRANSGENIC PLANTS

The plants obtained through genetic engineering contain a gene or genes usually from an unrelated organism, such genes are called transgenes and the plants containing transgenes are known as transgenic plants. Due to limitations of conventional breeding for attaining the desirable traits, the use of recombinant DNA technology has been taking advantage and development of transgenics.

Advantages of transgenic plants

The following are the advantages of transgenic plants:

1. Agronomic traits can be improved by producing plants with increased resistance to pest,
stress along with increased vigor, yield.
2. Enhanced efficiency of physiological process like photosynthesis and improved nutritional qualities.
3. Transgenic plants are also used as an analytical tool to explore aspects of gene regulation.
4. Major advantages of molecular breeding is that when a particular gene has been isolated and reconstructed in model plants and later it can be used in a large number of cultivars of different crops.

HOW TO MAKE TRANSGENIC PLANT

The whole process of introduction, integration and expression of foreign genes in the host is called genetic transformation (Figure 1). The understanding of the R gene function and resistance reaction have helped in designing the strategy for the development of transgenic plants resistant to different pathogens.

Gene transfer methods

Vector-mediated gene transfer

a. *Agrobacterium*-mediated gene transfer
b. DNA viruses as vectors

Vector less gene transfer

a. Direct uptake of DNA
b. Electroporation
c. Micro injection
d. Micro projectile bombardement

Vector-mediated gene transfer

Foreign genes are transported into recipient cells, protoplast or intact plant through a vector. It is a DNA molecule capable of replication in a host organism, into which a gene is inserted to construct a recombinant DNA molecule. This is also called indirect method of gene transfer. A vector could be either DNA virus such as caulimovirus or plasmid.

***Agrobacterium*-mediated gene transfer:** Among the various vectors used in plant transformation , the Ti plasmid of *Agrobacterium tumefaciens* has been widely used . This bacterium is known as “natural genetic engineer” of plants because it has natural ability to transfer T-DNA of its plasmids into plant genome upon infection of cells at the wound site and causes an unorganized growth of a cell mass known as crown gall. Ti plasmids are used as gene vectors for delivering useful foreign genes into target plant cells and tissues. The foreign gene is cloned in the T-DNA region of Ti plasmid. To transfer the foreign DNA in host plants, leaf discs, embryogenic callus or other suitable explants are collected and infected with *Agrobacterium* carrying recombinant disarmed Ti plasmid vector. In general, briefly the vector tissue is then cultured on a shoot regeneration medium for 2-3 days during which the transfer of T-DNA along with foreign genes takes place. After this, the transformed tissues are transferred onto a regeneration medium supplemented with usually lethal concentration of an antibiotic to selectively eliminate non-transformed tissues. After 3-5 weeks, the regenerated shoots are transferred to root -inducing medium, root shoots are acclimatized and after 3-4 weeks, complete plants are transferred to soil. Molecular techniques like polymerase chain reaction (PCR) and Southern hybridization are used to detect the presence of foreign genes in the transgenic plants.

DNA viruses as vectors: The genomes of caulimoviruses such as cauliflower mosaic virus (CaMv) and germinivirus such as tomato golden mosaic virus (TGMv) are double stranded DNA which makes these viruses as potential transformation vectors. Methotrexate resistant dihydrofolate reductive (DHFR) gene of *E. coli* has been successfully cloned into an intergenic region of CaMv. This engineered CaMv was used to infect turnip plants.

Vector less gene transfer

This is non-biological method for introduction of foreign genes into plants. Gene transfer in monocotyledonous plants is done by this method. Chemical and physical means are used to facilitate the entry of DNA into plant cells.

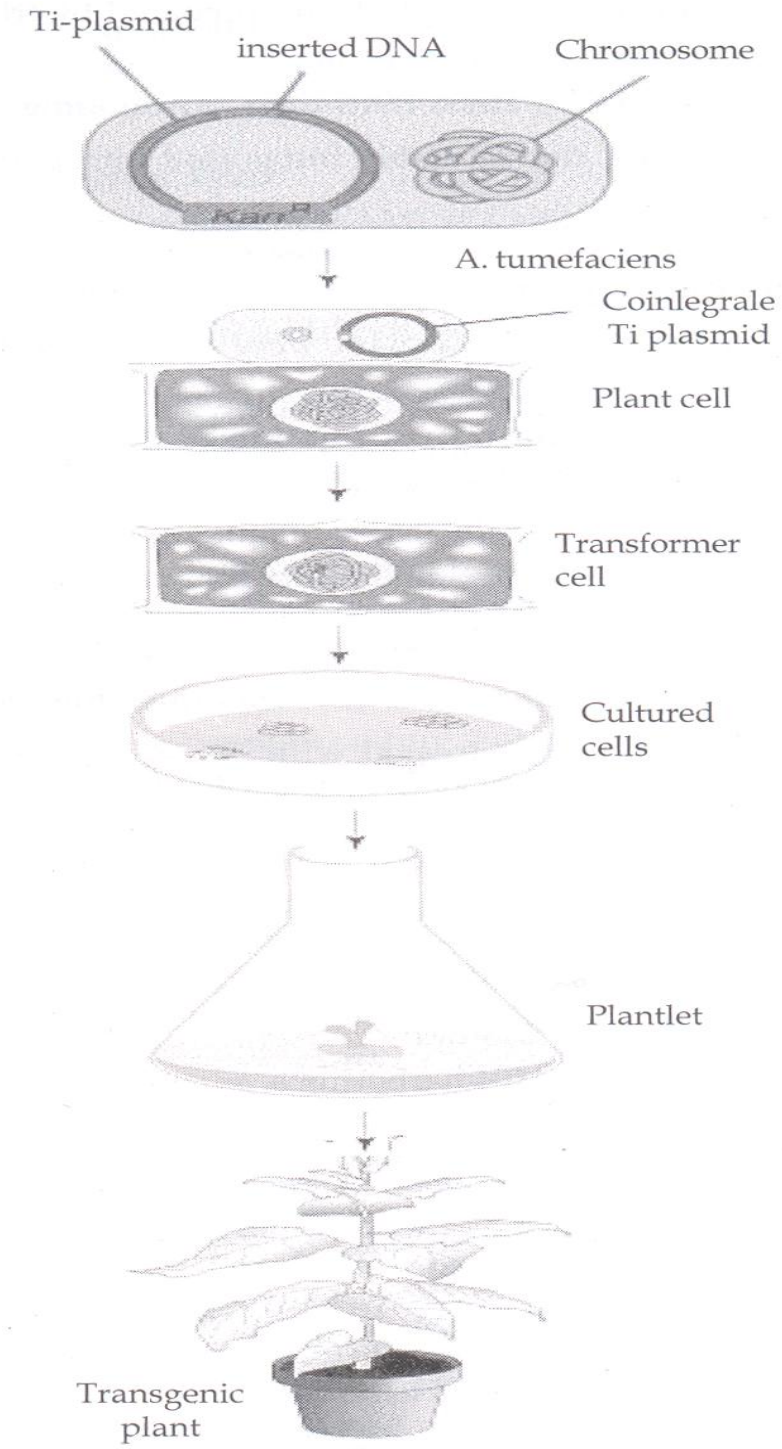

Figure 1. Schematic representaion of transfer of gene using ti-plasmid. Source: Rukamet et al., 2010.

Direct uptake of DNA: Direct DNA uptake by protoplasts can be stimulated by chemicals like polyethylene glycol (PEG) and dextran sulphate. PEG is used to improve the efficiency of electroporation. PEG at high concentration (15-25%) precipitates ionic macromolecules such as DNA and stimulate their uptake by endocytosis without any gross damage to protoplasts. This step is followed by cell wall formation and initiation of cell division. Now these cells can be plated at low density on selection medium.

However, there are problems in using this method for getting transgenic plants mainly related to plant regeneration from protoplasts and particularly in woody species.

Electroporation: This method is based on the use of short electric impulses of high field strength; a pulse of high voltage applied to protoplast/cells/tissues makes transient (temporary) pores in the plasma membrane which facilitate the uptake of foreign DNA, if the DNA is in direct contact with the membrane. The electroporation pulse is generated by discharging a capacitor across the electrodes in a specially designed electroporation chamber. Either a high voltage (1.5 kV) rectangular wave pulse of short duration or a low voltage (350 V) pulse of long duration is used. Using electroporation method, successful transfer of genes was achieved with the protoplast of tobacco, maize, rice, wheat and sorgum. In most of these cases, gene(s) associated with a suitable promoter sequence was transferred. Transformation frequencies can be further improved by using field strength of 1.25 kV/cm, adding PEG after adding DNA, heat shocking protoplasts at 45°C for 5 min before adding DNA and by using linear instead of circular DNA. For delivery of DNA to protoplasts, electroporation is one of the techniques most used for efficient transformation. However, since regeneration from protoplasts is not always possible, cultured cells or tissue explants are often used. Consequently, it is important to test whether electroporation could transfer genes also into walled cells.

Microinjection: The DNA is directly injected into plant protoplasts or cells (especially into the nucleus or cytoplasm) using fine tipped (0.5-1.0 micrometer diameter) glass needle or micropipette. This method of gene transfer is used to introduce DNA into large cells such as oocytes, eggs and the cells of early embryo. The process is observed and controlled under the microscope. The DNA is then integrated into the plant genome-probably during the cells own DNA repair processes. The advantages of microinjection is that, the target gene, which confers a new trait, is introduced directly into a single cell. The cells transformed in this way are easy to identify if a dye is injected along with the DNA. If the process works, it will no longer be necessary to select the transformed cells using antibiotic resistance or herbicide resistance markers.

Micro projectile bombardment: In recent years, it has been shown that DNA delivery to plant cells is also possible, when heavy micro particles (tungsten; or gold) coated with the DNA of interest are accelerated to a very high initial velocity (1,400 ft/s).These micro projectiles, normally 1-3 pm in diameter , are carried by a macro projectile or the bullet and are accelerated into living plant cells (target cells can be pollen, cultured cells in differentiated tissues and meristems) so that they could penetrate cell walls of intact tissue. The acceleration is achieved either by an explosive charge or by using shock waves initiated by a high voltage electric discharge. This technique has been used to produce stable genetic transformation in soybean, tobacco, maize, rice, wheat, among others. Transient expression of genes transferred in cells by this method has also been observed in onion, maize, rice, and wheat (Table 1).

ENGINEERING INSECT RESISTANCE

The most known approaches to develop insect resistant transgenic plants are introduction of bacterial gene Bt synthetic Bt and introduction of plant gene(s) for insecticidal proteins.

Introduction of bacterial gene Bt synthetic Bt

Bacillus thuringiensis synthesizes an insecticidal crystal protein which resides in the inclusion bodies produced by the *Bacillus* during sporulation. This crystal protein when ingested by insect larvae is solubilized in the alkaline conditions of the midgut of insect and processed by midgut proteases to produce a protease resistant polypeptide which is toxic to the insect. *Bt* lepidopteran specific from *B. thuringiensis* subsp.Kurstaki has been widely and successfully used in tobacco, tomato, potato, cotton, rice and maize for developing resistance against several lepidopteron insect pests. The use of redesigned synthetic *Bt* genes has also been used in some of these crops and in several instances the synthetic versions have exhibited up to 500- fold increase in the expression. Some of the transgenic have been released in the field for commercial cultivation.

Introduction of plant gene(s) for insecticidal proteins

Several insecticidal proteins of plant origin such as lectins, amylase inhibitors and protease inhibitors can retard insect growth and development when ingested at high doses. Some genes like CpTi, P1N-1, P1N11, A-1 and GNA have been cloned and being used in the transformation programme aiming at the insect resistance.

ENGINEERING FOR HERBICIDE RESISTANCE

There have been two approaches to develop herbicide resistant transgenic plants. Transfer of gene whose enzyme products detoxify the herbicide (detoxification) and transfer of gene whose enzyme product becomes insensitive to herbicide (target modification).

Table 1. Genetic transformation in monocotyledonous plants.

Work	Reference
Few transgenic rice plants by inoculating immature embryos with a strain of *A. tumefaciens*	Chua et al. (1993)
Reported a method for efficient production of transgenic rice plants from calli of japonica cultivars that had been co-cultivated with *A. tumefaciens*	Hiei et al. (1994)
The successful application of calli of japonica cultivars method to Basmati cultivars of indica rice	Rashid et al. (1996)
Achieved efficient transformation of maize by *A. tumefaciens*	Irshida et al. (1996)
Transformation of monocots and cereals	Heii et al. (1997)

Table 2. Herbicide resistant transgenic plants.

Species modified	Transgene source	Transgene product
Target modifications : *Betavulgaris*, *Nicotiana tobaccum*	*Arabidopsis thaliana*, *A. thaliana*	Acetolactate synthase, Acetolactate synthase
Enzyme overproduction : *Glycine max*	*Plant and microbial genes*	Analogue of EPSP synthase
Enzyme detoxification : *Brassica napus*	*Streptomyces hygroscopicus*	Phosphinothricin

Transgenic plants by Kakralya/Ahuja.

Transfer of gene whose enzyme product detoxify the herbicide (detoxification)

Using this approach, the introduced gene produces an enzyme which degrade the herbicide sprayed on the plants. Introduction of bar gene cloned from bacteria *Stroptomyces hygroscopicus* into plants, make them resistant to herbicides based on phosphinothricin (Table 2). Bar gene produces an enzyme, phosphinothricin acetyl transferase (PAT) which degrades phosphinothricin into a non-toxic acetylated form. Plants engineered with bar gene were found to grow in ppt at levels 4-10 times higher than normal field application. Bxn gene of *Klebsiella ozaenae* which produces nitrilase enzyme imparts resistance to plants against herbicide bromoxynil. Other genes including tfdA for 2,4-D tolerance and GST gene for Atrazine tolerance have also been used.

Transfer of gene whose enzyme product becomes insensitive to herbicide (target modification)

In this approach, a mutated gene is introduced which produces modified enzyme in the plant which is not recognized by the herbicide, hence the herbicide cannot kill the plant. A mutant aroA gene from bacteria *Salmonella typhimurium* has been used for developing tolerance to herbicide; glyphosate. Tolerance to herbicides has been achieved by engineering the expression of the mutant herbicide ALS gene derived from plant.

ENGINEERING VIRUS RESISTANCE

The genetic engineering of virus resistant plants has exploited new genes derived from viruses themselves in a concept referred to as pathogen derived resistance (PDR).

1. Coat protein mediated resistance (cP-MR).
2. Satellite RNAs mediated resistance.
3. Antisense mediated protection.

Coat protein mediated resistance (cP-MR)

Introduction of viral coat-protein gene into the plant, make the plant resistant to virus from which the gene for the cP was derived (Shah et al., 1995). It was 1st demonstrated for TMV in tobacco. Virus resistant transgenic have been developed in tomato, melon, rice, papaya, potato and sugar beet. Several cP-MR varieties of potato, cucumber and tomato are under field evaluation.

Satellite RNAs mediated resistance

Satellite RNAs are molecules which show little sequence homologies with the virus to which they are associated, yet are replicated by the virus polymerase and appear to affect the severity of infection produced by virus. It has been demonstrated that engineering cucumber, using cucumber mosaic virus (CMV) satellite RNA lead to transgenic resistant to CMV. This approach has been extended to several other crops.

Antisense mediated protection

It is now established that gene expression can be controlled by antisense RNA. cDNAS representing viral RNA genome were cloned in an antisense orientation to a promoter and transferal though the protection was not as effective as with coat protein. This approach has been effective against TMV gene.

Engineering for fungal resistance

There have been two approaches to develop fungal resistant transgenic plants.

1. Antifungal protein-mediated resistance.
2. Antifungal-compound mediated resistance.

Antifungal protein-mediated resistance

Introduction of chitinase gene in tobacco and rice has been shown to enhance the fungal resistance in plants. Chitinase enzymes degrade the major constituents of the fungal cell wall (chitin). Coexpression of chitinase gene in tobacco and tomato plants confers higher level of resistance alone. A radish gene encoding antifungal protein 2(Rs-AFP2) was expressed in transgenic tobacco and resistance to *Alternaria longipes* was observed.

Antifungal-compound mediated resistance

The low molecular weight compounds such as phytoalexins possess antimicrobial properties and have been postulated to play an important role in plant resistance to fungal and bacterial pathogens. Expression of a stilbene synthase gene from grapevine in tobacco resulted in the production of new phytoalexin (resveratrol) and enhanced resistance to infection by *Botrytis cinerea*. Active oxygen species (AOS) including hydrogen per oxide also play an important role in plant defense responses to pathogen infection. Transgenic potato plants expressing an H_2O_2 generating fungal gene for glucose oxidase were found to have elevated levels of H_2O_2 and enhanced levels of resistance both to fungal and bacterial pathogens particularly to *Verticillium wilth.*

BACTERIAL RESISTANCE

Genetic engineering for bacterial resistance has relatively met with little success. The expression of a bacteriophage T4 lysozyme in transgenic potato tubers led to increased resistance to *Erwinia caroto* Vora. Besides, the expression of barley a –thionin gene significantly enhanced the resistence of transgenic tobacco to bacteria *Pseudomonas syringae*. Advances in the cloning of several new bacterial resistance genes such as the *Arabidopsis*. RPS2 gene, tomato Cf9 and tomato P to gene may provide better understanding in the area of plant bacterial inter reactions.

Engineering for a biotic stress tolerance

Transfer of cloned genes has resulted in the transgenic which are tolerant to some a biotic stresses. For frost protection, an antifreeze protein gene from fish has been transferred into tomato and tobacco. Likewise, a gene coding for glycerol-3-phosphate acyltransferase from *Arabidopsis* has been transferred to tobacco for enhancing cold tolerance (Table 3).

Engineering for male sterility

The introduction of barnase gene results into male sterility whereas the introduction of barstar gene into another plant results into development of restorer line. The resulting hybrid is fully fertile. This system has been commercially exploited in maize and oilseed rape.

Engineering for food processing/quality

Using antisense RNA technology and inhibiting polygalacturonase, shelf- life can be extended in vegetable and fruits. High protein ‘phaseolin’ and Ama-1 genes have been introduced to heterologous systems. Introduction of provitamin A and earotene genes have resulted into the production of ‘golden rice’.

GENETIC ENGINEERING

Genetic engineering has recently made some intervention into mulberry research. Efficient protocols have been developed for direct plant regeneration from explants and insertion of desired genes into plant genome via *A. tumefaciens* and particle bombardment mediated methods (Bhatnagar et al., 2003). Functional evidence indicate that expression of Hva1 has shown landmark achievements in combating stress response (Chauhan and Khurana, 2011; Fu et al., 2007; Maqbool et al., 2002; Sivamani et al., 2000). Transgenic mulberry plants were therefore generated by over expression of barley Hva1 using *Agrobacterium* mediated transformation (Table 4) (Lal et al., 2008). Detailed physiological, biochemical, and molecular evidence indicated increased performance of transgenic mulberry plants when subjected to simulated salinity and drought conditions. Transgenic and non transgenic lines behave differentially under stress conditions and show better cell membrane stability, photosynthetic yield, less photo oxidative damage, and high relative water content under salinity and water stress than non-transgenic lines.

Table 3. Foreign genes expressed in transgenic plants.

Gene	Origin	Host	Stress
Bet A	*E. coli*	Tobacco	Salinity
Bet A	*E. coli*	Potato	Freezing
Mltd	*E. coli*	Arabidopsis	Salinity
Fad7	*Arabidopsis*	Tobacco	Chilling
HVA-1	Barley	Rice	Salinity and drought
Mn-SoD	*N.plumbaginif* olia	Alfalfa	Drought and freezing

Transgenic Plants by Kakralya/Ahuja.

Table 4. Transgenesis in mulberry for abiotic stress tolerance.

Gene	Expression profile	Reference
WAP21	Cold tolerance	Ukaji et al.(1999)
COR	Cold tolerance	Ukaji et al.(2001)
AlaBlb	Salinity tolerance	Wang et al. (2003**)**
OC	Insect resistance	Wang et al. (2003)
SHN1	Drought tolerance	Aharoni et al. (2004)
HVA1	Drought and salinity stress	Lal et al.(2008)
Bch	Drought and salinity stress	Khurana (2010)
NHX	Drought and salinity stress	Khurana (2010)
Osmotin	Drought and salinity	Das et al. (2011)

WAP21, Water collection plan; COR, cold on regulation; AlaBlb, soybean glycine gene; OC, osteocalcin; SHN1, schnurri from *Drosophila melanogaster*; HVA1, *Hevea braziliensis*, abiotic stress gene; bch-L inhibitor 2, aminobicyclo-(2,2,1)-heptane-2-carboxylic acid; NHX, Na+/H+ exchanger;Osmotin-Osmotic stress induced gene. Adopted from Vijayan et al., 2011.

Hva1 thus confers broad spectrum of tolerance against various a biotic conditions in transgenic plants. Preliminary studies regarding suitability of these transgenic plants for silkworm rearing was also performed. However, transgenic mulberry plants with barley hva1 gene under the control of CaMV35S promoter displayed growth retardation under normal conditions. Therefore, stress-inducible rd29A promoter was used instead of constitutive CaMV35S promoter for the overexpresssion of Hva1 to minimize the negative effects on plant growth. Results indicate that a combination of rd29a and hva1 is useful for tolerance against diverse stresses and minimizing negative effects on plant growth (unpublished data). Subsequently, efforts have been initiated for field trials of these transgenic mulberry lines for assessing their growth under field conditions. Similar success was achieved in genetic transformation of mulberry with tobacco osmotin under the control of a constitutive (CaMV 35S) as well as a stress-inducible promoter rd29A. Osmotin and osmotin-like proteins are stress proteins belonging to plant PR-5 group of proteins in several plant species in response to various types of biotic and a biotic stresses. Physiological analysis of transgenic plants under simulated salinity and drought stress as well as fungal challenge was undertaken to test the effect of the integrated gene. Transgenic plants with stress-inducible promoter were able to tolerate salt and drought stress efficiently than those with constitutive promoter, but in case of fungal tolerance 35S: osmotin transgenic plants performed better. Therefore, transformation of mulberry with the osmotin gene would confer tolerance against drought, salinity, and fungal pathogens (Das et al., 2011b). These transgenic plants were also well accepted by the silkworms—the ultimate users of mulberry leaves. Genetic transformation of *Morus indica* cv K-2 has also been achieved with bch1 (b-carotene hydroxylase-1), and screening of the transgenic mulberry revealed better tolerance of transgenic mulberry for high temperatures, high light, and UV radiation stress. These transgenic plants accumulate higher levels of xanthophylls under stress conditions than non-transgenic plants as revealed by ultra-performance liquid chromatography (UPLC). This is the first attempt of manipulating the carotenoid biosynthesis pathway in mulberry by over expressing b-carotene hydroxylase-1 gene (Das, 2009). Efficient screening method for mulberry was developed by Vijayan et al. (2003). In this method axillary buds were cultured *in vitro* saline conditions based on growth and development of shoots and roots under different salt concentration, salinity tolerance level of accessions were determined. Seed germination in a saline gel on petri-plates was also

used for identification of salt tolerant maternal parents (Vijayan et al. 2004).

Genetic transformation of mulberry

Agrobacterium mediated transformation has opened up several opportunities to develop mulberry transgenic. Progress made in developing transgenic at Delhi University (South campus) is quite impressive and has paved the way to initiate transgenic programmes in mulberry in other centres such as CSRTI, Mysore and UAS, Bangalore. Delhi University group has developed transgenic over expressing HVA1, a LEA_3 group stress responsive gene (Figure 2).

Over expression of HVA1 gene from barley generates tolerance to salinity and water stress in transgenic mulberry

The HVA1 gene from barley encodes a group 3LEA protein and is induced by abcissic acid (ABA) and water deficit conditions. Over expression of HVA1 in mulberry under a constitutive promoter via *Agrobacterium* mediated transformation. Molecular analysis of the transgenic plants revealed the stable integration and expression of the transgenic in the transformants. The transgenic plants showed better cellular membrane suitably, photosynthetic yield, less photo-oxidative damage and better water use efficiency as compared to non-transgenic plants under both salinity and drought stress. Amongst the lines analyzed for stress tolerance transgenic lines ST8 was relatively more salt tolerant, ST30 and ST31 was more drought tolerant (Lal et al., 2008).

Gene transfer by electroporation into protoplasts isolated from mulberry calli

For callus induction, explants were excised from the roots of mulberry seedlings grown in a medium containing benzylaminopurine, dichlorophenoxy acetic acid and thidiazuron. The isolated calli were repeatedly subcultured in the liquid medium resulting in a faster growing callus line. Protoplasts were enzymatically isolated from clumped cells and transfer of the β-gluconidas (GUS) gene by electroporation was carried out at various pulse voltages. Observation showed that successful transient expression of the GUS gene was accomplished in 20-30% of protoplasts at specific pulse voltage (Table 5).

Electroporation was conducted in various combinations of capacitance and pulse voltages, protoplasts stained by histochemical assay were observed constantly in pulse voltage ranging from 500-750 V/cm with a capacitance of 330 µFd .A typical result showed that survival rate of protoplasts decreased with increasing pulse voltage in electroporation. Transient expression of the GUS gene was operative in 20 -30% of protoplast electroporated at 500 and 750 V/cm. It was clearly demonstrated that the protoplasts from the callus line established by the study have the capability to permit successful transient expression of the GUS genes under the control of 35 S CaMV promoters, which is the first time this has been done in mulberry protoplasts. This success may open new possibilities in the analysis of various constructs using genes isolated from mulberry trees and in the production of transgenic mulberry clones as well.

Major improvements in gene delivery into mulberry leaf cells by particle inflow gun

The efficiency of gene delivery into mulberry leaf cells using a particle inflow gun for the production of b-glucronidase. Two days following bombardment with plasmid-coated micro projectiles, transiet expression of the b-glucuronidase was detected by forming a blue precipitate visually detectable within transformed cells. Bombardment efficiencies were determined by counting the number of blue spots that appeared in bombarded leaf tissue (Table 6). To survey the optimal tissue conditions for efficient bombardment, leaf tissue was precultured for various periods prior to bombardment with one µm micro projectiles. High levels of bombardment efficiency were obtained in day 0 – day 5 tissues after they were precultured. The prolonged preculture period markedly reduced bombardment efficiency.

Various treatments that could potentially enhance microprojectile penetration and gene expression were examined. Treatment with elevated osmoticum concentration may work in protecting the cells from leaking and bursting damage caused by micro projectile penetration. This idea was applied to the bombardment of mulberry leaf tissues. Treatment with 0.7 M manitol pre to bombardment tended to decrease the number of cells that transiently expressed the GUS gene (Table 7).

Bio-safety and risks of disease resistance transgenic plants

There is possibility of selectable marker gene (npt11) product to be toxic or allergic to human or animals. Mutation of sat RNAs may result in severe strain of the specific virus. Heteroencapsidation in case of cPMR may also result in development of virulent strain, recombination between engineered and challenged viruses.

Advantages of transgenic plants

Agronomic traits can be improved by producing plants

Figure 2. Drought and salinity tolerant transgenic mulberry.

Table 5. Transient expression of GUS gene in electroporated protoplasts.

Pulse voltage[1] (v/cm)	Survival (%)		Stained protoplasts (%)[2]
	Initial	After electroporation	
500	92	78	21
750	92	68	32
1000	92	52	0

[1]Capacitance; 330 µFd; [2]percentage of blue-stained protoplasts in 150-200 surviving protoplasts counted (Source : Yukio et al., 1999).

Table 6. Difference of bombardment efficiency among leaves precultured for various periods.

Experiment	Preculture period (days)	No. of blue spots/cm^2
1	0	98.7±40.00
	3	86.5±29.3
	6	70.0±33.5
	9	17.0±8.1
2	5	112.0±31.3
	10	11.3±6.6
	15	11.8±5.1

Fifteen leaves were harvested at different preculture periods and bombarded (Source: Yukie et al., 2000).

Table 7. Improvement in bombardment efficiency by various treatment of target leaves.

Treatment	No. of blue spots/cm^2
Control	89.6±49.1
0.7 M mannitol	59.3±41.5
Heat shock	79.1±15.8
1% DMSO	167.0±40.4

Twelve leave which were precultured for 5 days were used for each treatment and bombarded. (Source Yukie et al., 2000)

with increased resistance to pest, disease, stress along with increased vigour and yield. Enhanced efficiency of physiological process like photosynthesis and improved nutritional qualities. Major advantage of molecular breeding is that when a particular gene has been isolated and reconstructed in model plants, later it can be used in a variety of cultivars of different crops.

Conclusion

Genetic engineering offers a very promising alternative to the chemical disease management practices and a good supplement to the conventional plant breeding methods. Integration of transgenic technology in a total system approach will result in ecofriendly and sustainable means of diseases and insect pest management.

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Full Length Research Paper

Molecular characterization of cytochrome P450 1B1 and effect of benzo(a) pyrene on its expression in Nile tilapia (*Oreochromis niloticus*)

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Cytochrome P4501 (CYP1) family enzymes are most active in hydroxylating a variety of environmental contaminants including Polyaromatic Hydrocarbons (PAH), planar polychlorinated biphenyls and arylamines. CYP1B which belongs to the cytochrome P450 superfamily of genes, is involved in the oxidation of endogenous and exogenous compounds, and could potentially be a useful biomarker in fish for exposure to arylhydrocarbon receptors (AhR) ligands. In this study, a new complementary DNA (cDNA) of the *CYP1B* subfamily encoding *1B1* was isolated from Nile tilapia (*Oreochromis niloticus*) liver after intracoelomic injection with benzo (a) pyrene (BaP). The full-length cDNA was 2107 base pair (bp) long and contained a 5' noncoding region of 29 bp, an open reading frame of 1527 bp coding for 508 amino acids and a stop codon, and a 3' noncoding region of 551 bp, respectively. The deduced amino acid sequence of Nile tilapia CYP1B1 shows similarities of 79.7, 70.3, 65.7, 65.4, 65.0, and 63.7% with Plaice CYP1B1, Japanese eel CYP1B1, zebra fish CYP1B1, common carp CYP1B1, common carp CYP1B2 and Channel catfish CYP1B1, respectively. The phylogenetic tree based on the amino acid sequences clearly shows tilapia CYP1B1 and Plaice CYP1B1 to be more closely related to each other than to the other CYP1B subfamilies. Furthermore, real-time PCR was used for measuring BaP induction of CYP1B1 mRNA in different organs of tilapia (*O. niloticus*), using β-actin gene as internal control, and the results revealed that there was a large increase in CYP1B1 mRNA in liver (22.8), intestine (2.0) and muscles (1.3).

Key words: *Oreochromis niloticus*, benzo (a) pyrene, CYP1B1 cDNA, sequence analysis, real-time PCR.

INTRODUCTION

CYP enzymes constitute a unique superfamily of heme-containing proteins that are bound to the membrane of the endoplasmic reticulum and play a crucial role as an oxidation-reduction compound of the monooxygenase system. This system is involved in the oxidative metabolism of a wide variety of xenobiotics such as drugs, carcinogens, and environmental disrupters, as well as endogenous substrates such as steroids and fatty acids (Nebert

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and Gonzalez, 1987; Nelson et al., 1996). Most chemical carcinogens in the environment are chemically inert in them and require metabolic activation by CYP enzymes to exhibit carcinogenicity in experimental ani-mals and humans (Conney, 1982; Guengerich and Shimada, 1991). Previously, *CYP1A* enzymes were thought to be the only enzymes responsible for the metabolic activation of most carcinogenic PAHs to reactive electrophiles in mice, rats and rabbits (Conney, 1982).

However, recently *CYP1B* genes have been isolated from mammals and, in common with the *CYP1A* family, they are transcriptionally activated by PAH, and their protein products metabolise PAH (Savas et al., 1994; Zhang et al., 1998). Indeed, metabolism and carcinogenesis studies have recently shown *CYP1B1* to be a critical and necessary enzyme in the activation of several xenobiotics, most notably the PAH 7, 12-dimethyl- benzanthracene (DMBA) (Shimada et al., 1996; Buters et al., 1999), they reported that *CYP1B1* is located exclusively at extra hepatic sites and mediates the carcinogenesis of DMBA.

Shimada et al. (1996, 2002) also reported that *CYP1B1* participates with *CYP1A1* and *CYP1A2* in the activation of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin, benzo (a) pyrene and related carcinogens causing initiation of cancers in human and mice.

Phylogenetic analysis of CYP1 family sequences indicated that mammalian CYP1A and CYP1B lines diverged before the evolutionary emergence of mammals, suggesting the possible existence of CYP1B in fishes (Nelson et al., 1996). In a preliminary report, Godard et al. (2000) described the occurrence of CYP1B-like sequences in the fish species scup and plaice. Subsequently, the full-length plaice sequence was obtained and classified as a CYP1B1 (Leaver and George, 2000).

For carp (*Cyprinus carpio*), two CYP1B sequences have been submitted to GeneBank namely CYP1B1 and CYP1B2 (El-Kady et al., 2004a, 2004b). BaP, a member of the PAH family, is rapidly metabolized often into unstable byproducts such as epoxides, which have mutagenic and cytotoxic effects (Miller and Ramos, 2001). Thus, it elicit toxic effects at least in part by activating the AhR (Denison and Nagy, 2003), and AhR activation has been shown to affect the transcription of CYP isozymes 1A and 1B1 (Nebert et al., 2000). Therefore, in this study, the fish *Oreochromis niloticus* was used as a model organism to investigate the central hypothesis that CYP1B is involved in the molecular mechanisms of BaP-mediated toxicity.

Successful completion of this aim will provide a greater molecular understanding of this important P450 gene and its role in the mechanisms of action of PAHs. This research will further define the utility of *O. niloticus* fish as a model organism for studying PAH-associated toxicities. To achieve such a purpose, cDNA of the *CYP1B1* gene was isolated from the liver of *O. niloticus* fish after intracoelomic injection with BaP and sequenced. Phylogenetic analysis was performed to assess the relation-ship of this newly identified CYP1B1 gene with other CYP1B family members, and the expression pattern of CYP1B1 mRNA was determined in liver, intestine and muscle of tilapia using real -time PCR.

MATERIALS AND METHODS

Treatment of fish

Nile tilapia with a mean weight of 500 g were obtained from a local fish farm and were treated with a single intracoelomic injection of BaP (100 mg/ kg body weight) suspended in corn oil. Simultaneously, with the treated fish, control fish of similar mean weight was intracoelomicly injected with an equivalent volume of the vehicle (corn oil). The treated and control fish were killed 24 h after the injection and samples of liver, intestine and muscles were collected, immediately frozen in liquid nitrogen and stored at -80°C.

RNA isolation

Total RNA was isolated from 2 g of each of the samples of frozen liver, intestine and muscles according to the Standard Acid Guanidinium Thiocyante Phenol Choloroform (AGPC) extraction method (Chomczynski and Sacchi, 1978). Total RNA concentration and purity were determined spectrophotometrically as described by Sambrook and Russel (2001), and A_{260}/A_{280} ratio were between 1.7 and 1.9. Poly $(A)^+$ RNA was purified using an Oligotex-dt30 <super> mRNA purification kit (Takara, Japan).

Reverse transcriptase-assisted polymerase chain reaction

Reverse transcription of mRNA was performed with Superscript II reverse transcriptase (Gibco BRL,USA) to generate 5'-RACE-Ready and 3'- RACE-Ready first strand cDNA using a SMART™ RACE cDNA amplification kit (Clontech, USA) according to the manufacture's protocol.

Oligonucleotide primers and PCR amplification of tilapia *CYP1B1* cDNA fragment

Degenerate primers (Genenet.co.jp) were designed based on the conserved regions of four fishes CYP1B sequences retrievable from GenBank (Table 1). The sense primer (5'- GGR AGC ATM GTG GAY GT -3'; where R is for A or G; M for A or C and Y is for T or C) and the antisense primer (5'- GTG SGG RAT GGT KAS RGG -3'; where S is for G or C; R is for A or G, and K for G or T). PCR reactions contained cDNA (2.5 µl), 200 µM each of dNTPs, 100 µM each of degenerate primers, 0.25 µl Taq Polymerase Mix, and 10× reaction buffer in a final volume of 50 µl. The cycling conditions were as follows: Initial denaturation step at 94°C for 150 s followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min and ended by a final extension step at 72°C for 5 min. PCR products were analyzed on 1% agarose gels. The DNA band of expected size was excised with a scalpel, purified using GFX PCR DNA and a gel band purification kit (GE Health Care, UK), the PCR products obtained were cloned into PT7BlueT- vector (Novagen, USA). Purified plasmids were directly sequenced by dye terminator cycle sequencing using an ABI PRISM dye terminator cycle3130 xl DNA sequencer.

Table 1. *CYP1B* genes used in designing of degenerate primers.

Gene	Specie	Accession number
CYP1B1	common carp	AB048942
CYP1B2	common carp	AY437775
CYP1B1	Japanese eel	AY518340
CYP1B1	channel catfish	DQ088663

Table 2. Oligonucleotide primers used in PCR amplification of Tilapia CYP1B1 cDNA fragments.

Primer	Nucleotide Sequence	Nucleotide location
F1	5'- GGACGTTATGCCCTGGCTGCAGTA -3'	662-685
F2	5'- ACTTCCCCAACCCCATCAAAACCA -3'	685-708
R1	5'- CGGTGTAGCCCATGATGGATGTG-3'	1139-1161
R2	5'- TCCTGCTGGAGACGCACCTGTATC-3'	974-997

Table 3. Real-Time PCR primers of *Oreochromis niloticus* CYP1B1 and β-actin genes.

Gene	Primer description	Sequence (5'-3')	Location	Product size
CYP1B1	F	5'- TTACGTCATGGCCTTCATCTAC -3'	1058- 1079	122 bp
	R	5'- ATGACTGTGTTCTTTGGTACGG -3'	1159-1180	
β-actin	F	5'-GGGTCAGAAAGACAGCTACGTT-3'	42-63	143 bp
	R	5'-CTCAGCTCGTTGTAGAAGGTGT-3'	164-185	

3' and 5' RACE PCRs for full length cDNA

Four gene-specific primers (GSP) were designed based on the sequence obtained from PCR with degenerate primers. The primer pairs GSP-F1 and GSP-R1 (Genenet.co.jp) in combination with the universal primer mix included in a RACE PCR kit were used for the initial 5'- and 3'-RACE, respectively (Table 2). Initial 5'- and 3'-RACE PCR reactions were diluted by 50-fold using tricine EDTA buffer and a 5 µl aliquot of diluted PCR reactions was used in each of 50 µl nested-PCR reactions using primers GSP-F2 and GSP-R2 in combination with the nested universal primer mix included in a RACE PCR kit for 5'- and 3'-RACE, respectively. The initial RACE PCR reactions contained the same components as in the degenerate primer PCR except that the final concentration of each primer was 20 µM. PCR conditions were 5 cycles of denaturation for 30 s at 94°C and annealing for 3 min at 72°C; 5 cycles of denaturation at 94°C for 30 s, annealing at 70°C for 30 s, and extension for 3 min at 72°C and finally 35 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 30 s and extension at 72°C for 3 min. Nested-PCR reactions contained the same components as in the initial PCR except that diluted initial PCR products (5 µl) were used instead of the Race cDNA as the template and the nested universal primer (10 µM) was used instead of the universal primer mix.

The cycle conditions for the nested-PCR were as follows: 35 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 3 min. PCR products were purified, cloned and sequenced as previously described. Sequence has been deposited in the GenBank/NCBI data bank with an accession number HQ829968.

Phylogenetic analysis

DNA sequences with the following GenBank accession numbers were retrieved from the database and used in the phylogenetic analysis: AB048942 (common carp CYP1B1), AY437775 (common carp CYP1B2), AY518340 (Japanese eel CYP1B1), DQ088663 (channel catfish CYP1B1), AJ249074 (Plaice CYP1B1) and AY727864 (zebra fish CYP1B1). In order to determine homology among CYP1B family cDNAs or deduced amino acid sequences from various species, sequence alignment was performed by the CLUSTAL W method using Laser gene Megalign program (Ver 5.52,2003, DNASTAR Inc).

CYP1B1 expression in different organs of *Orochromis niloticus* using real -time PCR

Reverse transcription

Reverse transcription of the RNA samples isolated from liver, intestine and muscles was performed using Primescript™ RT reagent kit (Takara, Japan) according to the manufacturer's instructions. Reactions were incubated for 15 min at 37°C then 5 s at 85°C to inactivate the reverse transcriptase. RT products were stored at 4°C for further PCRs.

Primer design for real- time PCR reaction

Primers for *O. niloticus CYP1B1*cDNA and β-actin cDNA (accession no. EU887951), as an internal standard (Table 3) were designed using Laser gene primer select program (Ver5.52, 2003, DNASTAR Inc), with melting temperatures (T_m) ranging from 58 to 60°C, and amplicon lengths of 50 to 150 bp. Optimal programmed primer annealing temperatures were designed closely so that the optimal annealing temperatures were close enough to run all reactions under the same thermal parameters.

Table 4. Percent identities of deduced amino acid sequences of fish *CYP1B* gene subfamilies.

	Plaice CYP1B1	J.eel CYP1B1	Zebrafish CYP1B1	Common carp CYP1B1	Common carp CYP1B2	Ch.catfish CYP1B1
O. niloticus CYP1B1	79.7	70.3	65.7	65.4	65.0	63.7
Plaice CYP1B1		67.2	63.2	63.1	63.0	62.2
J.eel CYP1B1			64.0	64.5	64.6	62.1
Zebrafish CYP1B1				84.6	85.0	67.1
Common carp CYP1B1					92.3	67.6
Common carp CYP1B2						68.0

Real-time PCR conditions and analysis

Each PCR reaction consisted of 10 µl of SYBR® Premix Ex Taq™ II (2X), 10 µM of each primer, 2 µl of cDNA template and double distilled water to a final volume of 20 µl. Reactions were then analyzed on an ABI 7300 Real-Time PCR System under the following conditions: 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 3 min. All standard dilutions, no template controls and induced samples were run in triplicates. The fluorescence signals were measured at the end of each extension step. The threshold cycle (C_t) was determined for each sample using the exponential growth phase and the baseline signal from the fluorescence versus cycle number plots. To ensure that a single product was amplified, melt curve analysis was performed on the PCR products at the end of each PCR run. The amount of CYP1B1 mRNA, normalized to β-actin mRNA, was given by the formula 2-ΔΔCT; where CT is the threshold cycle indicating the fractional cycle number at which the amount of amplified CYP1B1 reached threshold. The ΔCT value is determined by subtracting the average β-actin CT value from the average CYP1B1 CT value. Then, the calculation of ΔΔCT involves subtraction of the ΔCT value of the calibrator (in our case the calibrator was average ΔCT value of control fish response in the BaP studies) from ΔCT value of each sample. Accordingly, CYP1B1 mRNA levels were reported as fold change in abundance relative to the average calibrator response.

Statistical analysis

The statistical differences between the groups were determined, and the data expressed as mean ± standard deviation. Excel (Microsoft, NY) were used to analyze the data, and Student's t test was used for the comparisons. A P-value <0.05 was considered significant. At least three determinations were carried out for each data point.

RESULTS AND DISCUSSION

Nucleotide sequence analysis of CYP1B cDNA

An important recent finding in fish is that they have CYP1B and now CYP1C genes, CYP1B was first cloned in plaice (*Pleuronectes platessa*) (Leaver and George, 2000), then cloned in carp (El-Kady et al., 2004a, 2004b), channel catfish (*Ictalurus punctatus*) (Kristine et al., 2006) and zebrafish (Hou-Chu Yin, 2008). In both plaice and channel catfish, only a single isoform of CYP1B has been identified, whereas both CYP1B1 and CYP1B2 genes have been cloned in carp (*Cyprinus carpio*). In this study, a new cDNA of the CYP1B subfamily encoding CYP1B1 was isolated from Nile tilapia. The nucleotide sequence (Figure 1) contained a 5' noncoding region of 29 bp, an open reading frame of 1527 bp coding for 508 amino acids and a stop codon, and a 3' noncoding region of 551 bp. The predicted molecular weight was 57.67 KDa. The sequence had one polyadenylation signal (AAGAAA) and a poly A tail of 30 nucleotides. This sequence was aligned with the previously mentioned sequences by CLUSTAL W (Thompson et al., 1994) using Lasergene Megalign program, version 5.52, 2003 (DNASTAR Inc).

Comparison of amino acid sequences

Table 4 shows the percent identities of deduced amino acid sequences of *O*. *niloticus* CYP1B1 with the other fish CYP1B genes. The highest identity was 79.7% with plaice CYP1B1, followed by 70.3% with Japanese eel CYP1B1, 65.7% with zebra fish CYP1B1, 65.4% with common carp CYP1B1, then ended by 65% with common carp CYP1B2 and 63.7% with channel catfish CYP1B1. These results suggested that the obtained amplification product corresponds to tilapia CYP1B1 as it has more than 55% amino acid identity with other fishes CYP1Bs.

Phylogenetic analysis

The phylogenetic tree based on the amino acid sequences were used to assess the relationship of CYP1B1 of *O*. *niloticus* with those of other fish species.

Figure 2 clearly shows tilapia CYP1B1 and plaice CYP1B1 are more closely related to each other than to the other CYP1B subfamilies. As sequences from other species accrue, it is clear that many teleost fish possess a complement of three CYP1 subfamilies, CYP1A, CYP1B and CYP1C, and that the CYP1Bs and CYP1Cs together constitute a sister clade to the CYP1As (Godard et al., 2005).

Figure 1. Nucleotide sequence (2107 bp) of cytochrome CYP1B1 cDNA and its deduced amino acids (509) residues. Consensus sequence for polyadenylation signal (AAGAAA) is in bold. The stop codon, TAA, is marked with an asterisk.

Characteristic structural features of *Oreochromis niloticus* CYP1B1 protein

Substrate recognition sites (SRSs)

Sequence alignment of tilapia (*O. niloticus*) CYP1B1 protein with those of common carp CYP1B1 (accession no. AB048942), common carp CYP1B2 (accession no. AY437775), Plaice CYP1B1 (accession no. AJ249074), Japanese eel CYP1B1 (accession no. AY518340), channel catfish CYP1B1 (accession no. DQ088663), zebra fish CYP1B1 (accession no. AY727864) indicated

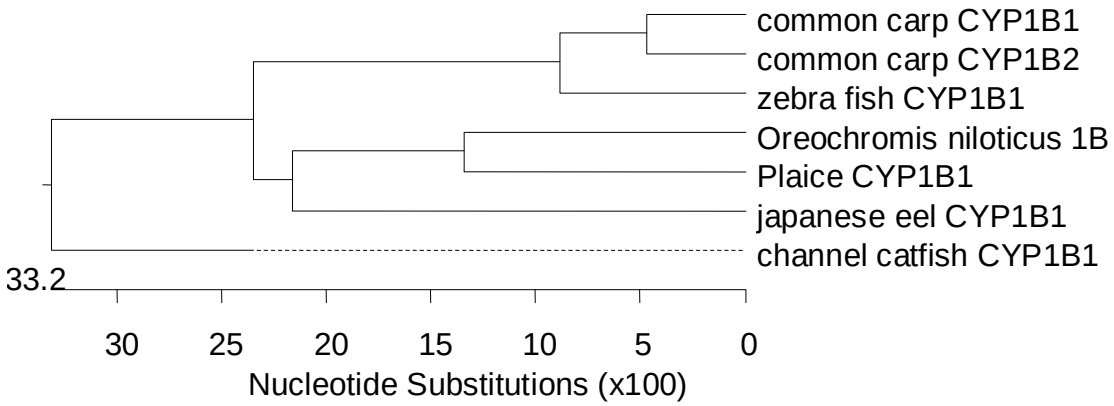

Figure 2. Phylogenetic tree of fish CYP1B1 cDNAs using the amino acid sequences of fishes.

that *O. niloticus* protein contain six separate substrate recognition sites (SRSs) (Figure 3). Location and amino acids sequences of the substrate recognition sites (SRSs) according to Gotoh (1992) are indicated as follows (Table 5).

Highest structural conserved regions: Deduced amino acid sequence of *O. niloticus* CYP1B1 possesses all major functional domains characteristics of previously discovered CYP1B1 molecules including (Figure 3):

1) A putative heme-binding cysteine at position 484 in the typical signature sequence of 477- FSLGKRRCIG-486, this sequence is highly conserved among teleost CYP1B1s, but an F477Y change (at position 477) exists in channel catfish CYP1B1. Also, L479M (at position 479) changed to M in channel catfish CYP1B1 and common carp CYP1B2 but changed to V (L479V) in common carp CYP1B1.
2) Threonine residue present in the I-helix at positions 348 in a signature sequence 344-ASQDT-348, this conserved threonine is proposed to play a crucial role in the binding of oxygen.
3) The proline rich region which is postulated to be crucial for the correct conformation of microsomal CYPs is found in the region downstream of the amino-terminal signal anchor sequences in a signature sequence 70-PPGPLAWP-77.
4) Arginine residue which is integral to enzymatic function in a signature sequence of 448-WSQPDIFDPERF-459.
5) The absolutely conserved Glu-X-X-Arg motif in helix K, this region is probably needed to stabilize the core structure.

CYP1B1 mRNA level in different tissues of BaP treated fish

PAHs are belonging to persistent organic pollutants (POPs), which have become the focus of concern because of their ever-growing level in the environment and wildlife. PAHs mainly result from incomplete combustion of organic materials and present in the air, soil and water (Gelboin, 1980; Chu et al., 2003; Chen et al., 2004). BaP, the most potent carcinogen in the PAH family, is the unique recognized carcinogen (a group 1 carcinogen) by the International Agency for Research on Cancer (IARC, 1983; Straif et al., 2005). BaP can be metabolically activated by CYP enzymes and epoxide hydrolase to form DNA adducts, thus exerting its mutagenic and carcinogenic effects. In the meantime, the metabolic enzymes are also involved in the degradation and final elimination of BaP. Therefore, the equilibrium and modulation of the metabolic enzyme level are of great significance to determine the damaging effects of BaP under different micro environments (Nahrgang et al., 2009; Vondrácek et al., 2009; Shi et al., 2010). Therefore, in this study, expression patterns of CYP1B1 mRNA were determined in liver, intestine and muscle of tilapia after intracoelomic injection of BaP using real -time PCR. Results revealed that there was a large increase in CYP1B1 mRNA in liver (22.8), intestine (2.0) and muscles (1.3) (Tables 6, 7 and Figure 4). The liver was chosen for its prominent role in xenobiotic and endogenous substrate (for example, steroid) metabolism by CYPs. Also, the induction of CYP1B1 in liver and intestine provided a defensive mechanism against the pollutants entering from the external environment. Concerning with CYP1B1 expression levels in other fish species, El-kady et al. (2004a) stated that carp exposed to 3- methylcholanthrene had CYP1B1 messenger RNA (mRNA) expression in liver, intestine and gill.

Kristine et al. (2006) study the induction of CYP1B mRNA expression in BaP-exposed catfish (20 mg/kg intra peritonealy after 4 days) and found that BaP exposure significantly induced CYP1B message in blood (10.7 fold), gonad (17.4 fold) and liver (13 fold) of laboratory catfish. Also, Wolinska et al. (2011) evaluated lethal and sub-lethal effects of BaP on mRNA expression of CYP1B1 in zebrafish (Danio rerio) larvae exposed for 48 h to a BaP concentration of 0.50 µmol•l, transcript quantification performed on the pools of zebra fish larvae revealed signi-

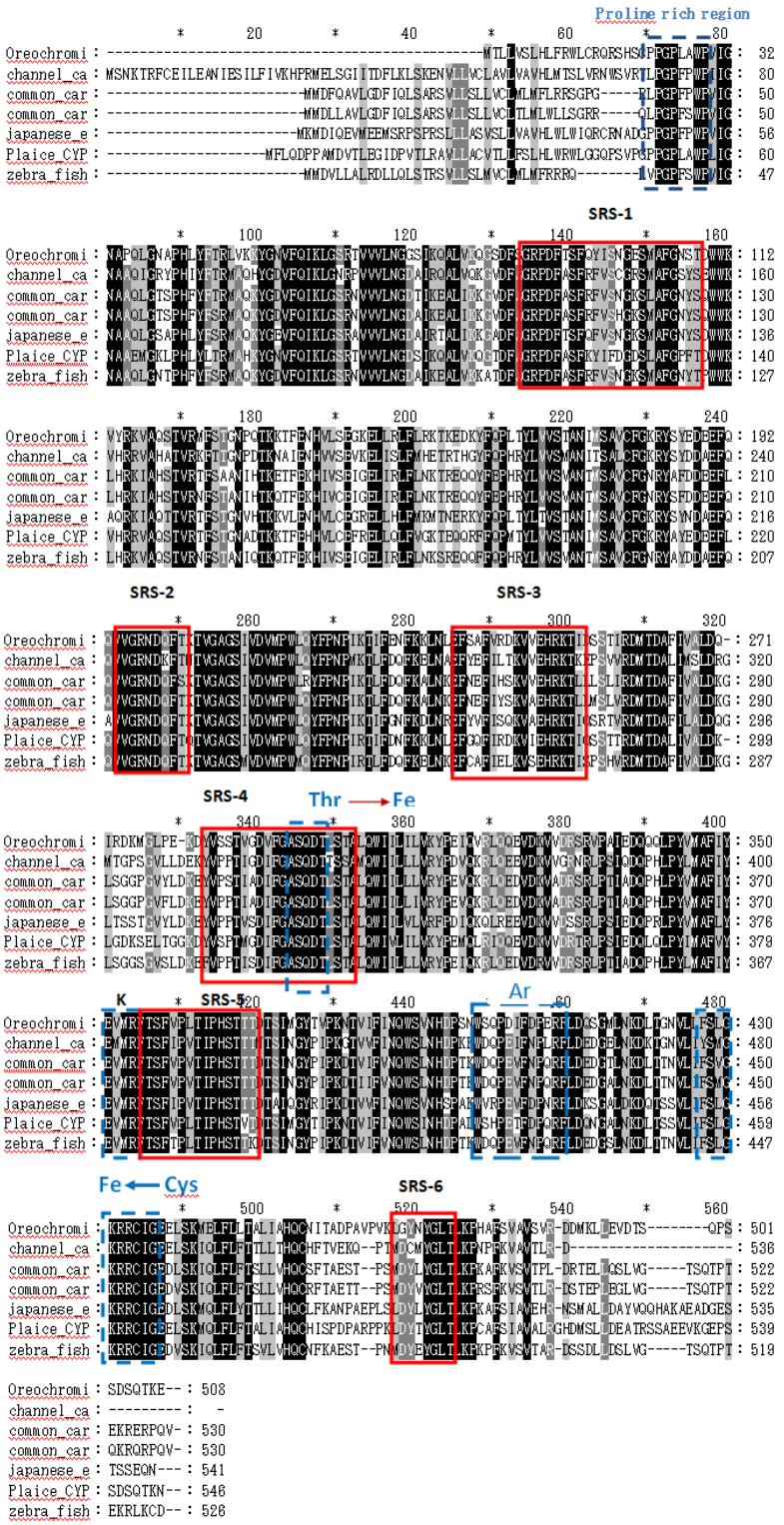

Figure 3. Sequence alignment of Tilapia CYP1B1 protein with those of other fishes CYP1B1 and CYP1B2. The sequences were aligned using Clustal W. The amino acid sequences in the red boxes indicate the positions corresponding to the Substrate Recognition Sequences (SRSs) in CYP1B. The amino acid sequences in the blue dashed boxes indicate proline rich region, K region, conserved threonine residue, Arginine residue and a putative heme-binding region respectively.

Table 5. Location and amino acid sequences of the six SRSs in *oreochromis niloticus* CYP1B1protein.

	Location		Amino acid sequence
	from	To	
SRS -1	134	156	GRPDFTSFQYISNGESMAFGNST
SRS-2	243	251	VGRNDQFT
SRS-3	286	301	FSAFVRDKVVEHRKTI
SRS-4	333	351	YVSSTVGDVFGASQDTLST
SRS-5	406	419	TSFVPLTIPHSTTT
SRS-6	517	524	LGYNYGLT

Table 6. Real time PCR results for Tilapia CYP1B1 and β-actin gene.

Sample name	Tilapia CYP1B1				β- actin gene			
	Mean C_t	Std Dev C_t	t-value	Pr	Mean C_t	Std Dev C_t	t-value	Pr
L. cont	30.2[a]	0.88	8.77	<0.005	18.80a	0.17	1.34	>.0.025
L. ind	25.92[b]	0.4			19.03a	0.23		
I. cont	28.78[a]	0.81	3.1	>0.025	17.54[a]	0.1	5.52	≤ 0.005
I .ind	27.33[a]	0.07			17.06[b]	0.11		
M. cont	21.33[a]	0.12	10.21		17.91[a]	0.15	4.23	
M. ind	23.48[b]	0.34		<0.005	19.64[b]	0.69		<0.025

Table 7. Amount of CYP1B1 mRNA, normalized to β-actin mRNA.

Sample name	Tilapia CYP1B1 average C_t	β-actin gene average C_t	ΔC_t	$\Delta\Delta C_t$	$2^{-\Delta\Delta Ct}$
L. cont	30.20	18.80	11.40	-4.51	22.8
L.ind	25.92	19.03	6.89		
I. cont	28.78	17.54	11.24	-0.97	2.0
I.ind	27.33	17.06	10.27		
M. cont	21.33	17.91	3.42	0.42	1.3
M. ind	23.48	19.64	3.84		

Where: L.cont: Liver control, L.ind: Liver induced, I.cont: Intestine control, I.ind: Intestine induced, M.cont: Muscle control M. ind: Muscle induced.

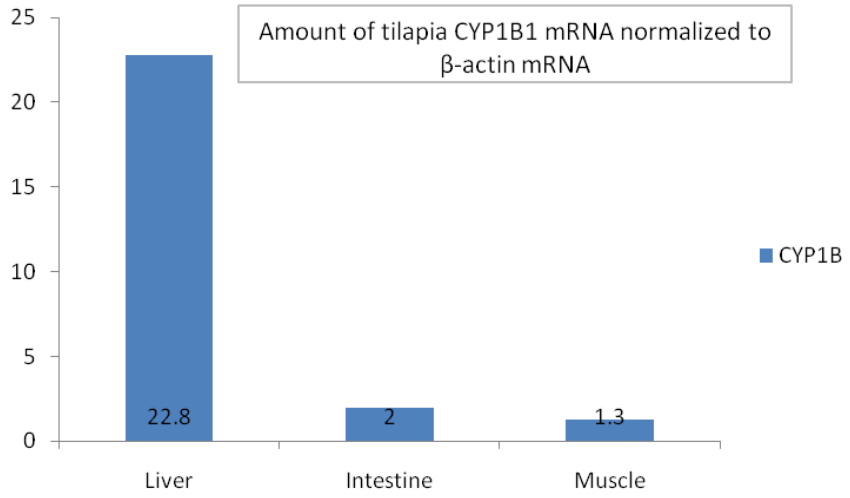

Figure 4. Tilapia CYP1B1 mRNA normalized to β-actin mRNA.

ficant mRNA accumulation [ER = 26.11 ($p<0.001$)]. The expression of CYP1B1 gene is regulated by AhR, which forms an active transcription factor heterodimer with the AhR nuclear translocator (ARNT) after ligand-binding such as BaP, and consequently induces the expression of the CYP1B1. Then, BaP is metabolized to form biologically active 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo (a) pyrene (BPDE) which can form several kinds of adducts with DNA (Nahrgang et al., 2009; Vondrácek et al., 2009). These adduct may subsequently lead to replication errors or mutations if not repaired in time.

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Full Length Research Paper

Isolation of microsatellite markers for *Bletilla striata* and cross-amplification in other related species

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Bletilla* is a temperate, terrestrial genus of orchids containing 6 species. For the species whose whole genome is unknown, we used magnetic bead hybridization method to develop microsatellite Simple Repeat Polymorphoresis (SSR) for *Bletilla striata* and 9 primer sets were characterized in two wild populations of *B. striata* and one wild population of *Bletilla ochracea.* The number of alleles per locus ranged from 1 to 12. The expected and observed heterozygosities ranged from 0 to 0.7646 and 0 to 0.950 in *B. striata*, respectively. In *B. ochracea*, the expected and observed heterozygosities ranged from 0.296 to 0.871 and 0.05 to 1, respectively. The 9 pairs of primers we designed can be used to distinguish different ecotypes and species, and might be used for other subspecies or species in genera *Bletilla.

Key words: *Bletilla striata*, cross-species amplification, simple repeat polymorphoresis (SSR).

INTRODUCTION

The genus *Bletilla*, composting about six species, is endemic to Asia with a distribution pattern from N Myanmar and Indochina through China to Japan. The usage of the plants of *Bletilla*, mainly *B. striata* (Thunb.) Reichb. f., includes art (Chinese painting and writing), the production of porcelain, and medicine (Lawler, 1984), as well as vegetable dyes for dyeing cloth in some minority people in Guizhou and Yunnan Province, China (Luo, person observation). Those species are also commonly used as a horticultural subject covering both indoors and out cultivation in North American and European areas (Pridgeon, 2005). Recently, *B. striata* has been reported to be able to suppress various weed species (Sakuno et al., 2010). As the traditional herb medicine using more than 1500 years, pseudobulbs of *Bletilla* have been widely used in Eastern Asian countries to treat alimentary canal mucosal damage, ulcer, bleeding, bruises and burns. The pseudobulbs also show antibacterial, anti-inflammatory, antiphlogistic and demulcent properties, and thus use in treating pneumonophthisis, pneumonorrhagia, tuberculosis and haemorrhage of the stomach or lung (Wang et al., 2006). Moreover, it is suggested that cationic polysaccharide from *B. strata* can serve as a non-viral nucleotide drug delivery vehicle for oligonucleoide or siRNA targeting to immunology system (Dong et al., 2009). Apparently, there is a huge potential demand for the pseudobulbs of *Bletilla* in future. At present, the cultivation of *Bletilla* is only limited to the temperature areas of North America and Europe for the horticultural purpose (Pridgeon, 2005), and the using of

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pseudobulbs of *Bletilla* for other purpose especially for medicinal purpose is basically based on wild resources.

Undoubtedly, the wild resource of *Bletilla* will rapidly decrease in Eastern Asian Countries due to over collections as well as habitat destruction and fragmentation (Chun and Chun, 2005). The conflict between the great market demand for the pseudobulbs of *Bletilla* and the decreased wild resource has necessitated the breeding of cultivars for high yields, high concentrations of active components and high resistance to diseases. Unfortunately, no cultivar has been bred so far and selections of superior individuals meeting the requirements are now underway from field and hybrid progenies. Molecular marker – assisted selection has become routine in cultivar breeding, and great efforts have been made to develop molecular markers such as microsatellites. More important, microsatellite markers have often been used for genetic diversity studies due to their desirable genetic attributes like hyper-variability, wide genomic distribution, co-dominant inheritance, reproducibility, multi-allelic nature and chromosome specific location (Singh et al., 2010; Tang et al., 2012). Here, we describe microsatellite loci which developed from *B. striata* and test the transferability of those markers to other related species. These loci were useful for further breeding superior cultivars and studies of genetic diversity, and contribute to knowledge on conservation of genus *Bletilla.*

MATERIALS AND METHODS

Genomic DNA of leaves of *B. striata* sampled from valley in Guiyang City, Guizhou Province, China (26° 29′ 46″ N, 106° 39′ 39″ E) was extracted using a plant genomic DNA Kit (Tiangen, Beiing, China). First, total genomic DNA was completely digested with the *Mse*I restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA), and then ligated to an *Mse*I adapter pair (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) with T4 DNA ligase (New England Biolabs) in a 30 µL reaction mixture. Tenfold diluted digestion–ligation mixture was amplified with adaptor-specific primers *Mse*I-N (5′-GATGAGTCCTGAGTAAN-3′) in 20 µL reactions with the following conditions: 3 min denaturation at 95°C; followed by 26 cycles of 30 s denaturation at 94°C, 1 min annealing at 53°C, and 1 min extension at 72°C; with a final extension of 72°C for 5 min and then were enriched for microsatellites with a 5′-biotinylated $(AG)_{15}$ probe. Thirdly, using adaptor-specific primers amplified enriched fragments again and using a PCR Purification Combo Kit (BioTeke, Beijing, China) purified it. Finally, the cleaned DNA fragments were ligated into the pEASY-T1vector (Promega, USA), and transformed into competent cells of trans1-T1 Phage Resistant Chemically (Transgen, Beijing, China). Hundreds of positive clones amplified by PCR, and 95 of these, with a size range of 500 to 1000 bp, were sequenced with an ABI PRISM 3730 DNA sequencer (Applied, Biosystems, USA). The sequences which contained SSR were designed specific primers using primer primer 5.0 (Lalitha, 2000) and thirty had sufficient regions to design primer.

All primer pairs were assayed in individuals PCR and run on 1% agarose gels. Thirteen primer pairs have specific and right loci. Then, to utilize fluorescently labeled M13 primer for sequencing, the forward primer of each pair added an M13 sequence (5′-TGTAAAACGACGGCCAGT-3′) to its 5′ (Schuelke, 2000). Using the fluorescently labeled primer for amplification, nine primer pairs showed clear bands and expected size. The 25 µl volumes PCR mix contained 4 pmol reverse primer and FAM/ HEX/ TAMRA-M13(-21) primer, 1 pmol of the forward primer, 12.5 mm Tris-HCl (pH 8.3), 62.5 mm KCl, 1.875 mm $MgCl_2$, 0.25 mm of each dNTP, 0.75 U DNA polymerase (TakaraTaq) and 50 ng template DNA. PCR amplification were done as follows: 94°C for 5 min, then 30 cycles at 94°C for 30 s, specific annealing temperature (Table 1) for 45 s, 72°C for 45 s, followed by 8 cycles at 94°C for 30 s, 53°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min. The nine microsatellite loci were characterized using 54 wild samples were collected from three Sites in China: 14 samples of *B. striata* from valley in Guiyang, Guizhou, POP- *GY*: 26° 29′ 46″ N, 106° 39′ 39″ E; 20 samples of *B. striata* from valley in Xinning, Hunan, POP-*XN*: 26° 25′ 42″ N, 110° 50′ 30″ E; 20 samples of *B. ochracea* from valley in Qingzhen, Guizhou, POP-*QZ*: 26° 36′ 2″ N, 106° 28′ 16″ E. The PCR amplification was same to the aforementioned described. Nine primer pairs tested for polymorphism on an ABIPRISM 3730 Genetic Analyzer and using GeneMapperv4.0 software (Applied Biosystems) analyzed the result.

The numbers of alleles per locus, observed and expected heterozygosities, and deviations from Hardy–Weinberg equilibrium (HWE) were estimated using FSTAT version 2.9.3 (Gouldet, 2001).

RESULTS

The newly developed microsatellite loci showed high levels of polymorphism in *B. striata*. The microsatellite markers can be used to evaluate the genetic diversity and infer evolutionary processes in natural populations, which should be useful for developing the appropriate conservation strategies for *B. Striata*.

DISCUSSION

Microsatellite markers are based on the amplification of internal sequences between microsatellites [intersimple sequence repeat (ISSR) markers] using PCR and then looking for microsatellite loci contained within these sequences, taking into account that microsatellites are generally clustered within the plant genome, and microsatellite markers have been proven useful in assessing genetic diversity of populations in different species (López-Roberts et al., 2012; Caitlin et al., 2013). In the present study, the result can draw that BJ67 were monomorphic in these two *B. striata* population, but polymorphic in *B. ochracea.* BJ311 and BJ120 failed to amplify in *B. ochracea*. In *B. striata* and *B. ochracea*, the number of alleles per locus ranged from 1 to 12 and the mean number of alleles per species was 4.33 and 4.14, respectively (Table 2). The expected and observed heterozygosities ranged from 0 to 0.7646 and 0 to 0.950 in *B. striata*, respectively. In *B. ochracea*, the expected and observed heterozygosities ranged from 0.296 to 0.871 and 0.05 to 1, respectively. The 9 pairs of primers we designed can be used to distinguish different ecotypes and species, and might be used for other subspecies or species in genera *Bletilla*, which would provide for implementing concrete protection strategy and/or tagging the very right medical herbs.

Table 1. The characteristics of nine microsatellite loci in *B. striata.*

Locus	Primers sequences (5-3′)	Repeat motif	Size range (bp)	Ta (°C)	GenBank accession No.
BJ109	F: CTATTATTCCTCCTCGTTTG R: CTAGCCTACCAAGTAGTTCC	$(GA)_{13}$	160-174	54	JQ965919
BJ98	F: GGCTAACCCATAATTGATC R: CTTTCATTGAGGTGGACTT	$(GA)_{23}$	258-310	58	JQ965917
BJ70	F: CACGAACAGCCACTATCA R: TTACAAGCCTCCCAATCT	$(GA)_{8}$	271-301	58	JQ965914
BJ120	F: CCATTACCAACCGTGGAG R: GTCGGACGAAAGTGAGCC	$(GA)_{10}$	112-126	58	JQ965916
BJ311	F: CCAAAGTGATAACGGAAGG R: TTGAATCCAAGAAGTGCC	$(GA)_{11}$	343	54	JQ965913
BJ303	F: TCAGTTTGTCGCTTCTATG R: ATCCTGAATCTGGGGCTA	$(TC)_{8}$	111-131	54	JQ965918
BJ67	F: CCGATGTGGAGGTAGAGC R: CGGAAACGGAAGAAGAAG	$(TTC)_{5}$	237	56	JQ965915
BJ68	F: CAAAGCAAACTGGACGAA R: CCATAATCACTTGGAACCC	$(GA)_{8}$	129-133	54	JQ965921
BJR100	F: GCTGAGGACAGAAGGGAG R: AGTAGAAATCATCGCACAA	$(AG)_{10}$	298-304	58	JQ965920

Table 2. Results of initial primer screening in two populations of *Bletilla striata* and one populations of *Bletilla ochracea*. Shown for each primer pair are the number of Alleles (A), average observed (H_o) and expected heterozygosity (H_e).

Locus	*POP-GY (N = 20)*			*POP-XN (N = 14)*			*POP-QZ (N = 20)*		
	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
BJ68	2	0.300	0.262	2	0.0714	0.0714	3	0.700	0.555
BJ98	6	0.950	0.751	7	0.5714	0.6799	12	0.800	0.871
BJ109	3	0.450	0.676	6	0.5714	0.7646	3	0.050	0.432
BJ303	2	0.350	0.296	2	0.1429	0.1376	5	0.450	0.706
BJ311	1	0	0	1	0	0	na		
BJ67	1	0	0	1	0	0	2	0.350	0.296
BJ70	2	0.550	0.409	4	0.3571	0.5608	2	0.500	0.431
BJ120	2	0.750	0.481	5	0.7143	0.7249	na		
BJR100	2	0.850	0.512	4	0.8571	0.5767	2	1.000	0.513

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Full Length Research Paper

Molecular cloning and characterization of glucose transporter 1 (glut1) and citrate synthase cDNA in buffalo (*Bubalus bubalis*)

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Glucose transporter type-1 (glut1) and citrate synthase plays crucial role in glucose transport and regulation of tricarboxylic acid cycle (TCA) cycle in mammalian energy metabolism. The present study was aimed to clone and characterize glut1 and citrate synthase cDNA in water buffalo (*Bubalus bubalis*). Total of 90 cumulus oocyte complexes (COCs) were used for mRNA isolation and reverse transcribed to cDNA, which was further used in polymerase chain reaction (PCR) amplification of glut1 and citrate synthase. PCR products of glut1 and citrate synthase were cloned by T/A cloning using pGEM-T easy vector and further sequenced. Gene sequence analysis of glut1 and citrate synthase revealed that they have open reading frame of 1479 (encoding 492 aa) and 1401 bp (encoding 466 aa), respectively. Further phylogenic analysis of gene and deduced amino acid sequences suggests that bubaline glut1 shares ~ 89 to 98% and ~ 97 to 99% similarity at nucleotide and amino acid level respectively whereas citrate synthase shared ~ 89 to 99% at nucleotide and ~ 96 to 99% at amino acid level respectively with other domestic species and human. Predicted protein structures of buffalo glut1 protein accentuate the presence of crucial amino acids involved in glucose transport moreover the essential catalytic residues are highly conserved in buffalo citrate synthase.

Key words: Buffalo, cloning, characterization, Glut1, citrate synthase.

INTRODUCTION

Buffalo (*Bubalus bubalis*) constitutes the major pillar of the dairy and meat industry of India contributing a large share to agricultural gross domestic product (GDP), maintenance and improvement of such an important domestic species requires an elaborate database. The gene sequence data, sequence homology and phylogenetic relationship with different domestic species clearly depict the functional differences at gene and protein level. Citrate synthase is a key regulatory metabolic enzyme that catalyzes the first step in tri-carboxylic acid (TCA) cycle, a potential regulator of aerobic energy production in highly dividing cells and is present in virtually all the cells capable of oxidative metabolism, therefore, a critical enzyme in cellular biosynthesis (Weitzman et al., 1976). Porcine cardiac tissue derived form of citrate synthase is most widely studied (Bloxham et al., 1981 and Evans et al., 1988) and it has now been isolated from numerous sources. Most common form of citrate synthase consists a dimer of molecular weight 90,000 to 100,000 (Srere, 1975; Singh et al., 1970) however, a tetrameric form with larger subunit of molecular weight (55,000 to 60,000) was also reported in some prokaryotic organisms (Weitzman, 1976). Bovine heart derived citrate synthase cDNA sequence encodes a 466 amino acids containing protein

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Table 1. Oligonucleotide primers for gene specific RT-PCR amplification.

Gene of interest	Primer sequence	Annealing temp (°C)	Fragment size (bp)	Reference /Gene bank accession number
Glut-1 primer1	5'AGAGGGAGGCCAAGAGAGTC 3' CCTTCACTGTCGTGTCGCTA	54	1091	M60448.1
Glut-1 primer 2	5'GTCACCATCCTGGAGCTGTT 3' TACCCCAAGAGGTGGCTATG	54	1161	M60448.1
Citrate synthase	5'GCCATGGCTTTACTCACTGC 3' CCTCCCCATCTTCAGTTTCA	60	1421	NM_001044721.1
β-Actin	5'GACGATGCTCCCCGGGCCGTCT 3'ATGGGGTACTTGAGGGTCAGGA	51	143	BT030480

and it shares 95.1 and 96.3% similarity with human and porcine sequences respectively (Winger et al., 2000). Earlier reports showed that the alteration in several catalytic residues within citrate synthase leads to decrease in catalytic efficiency of the enzyme (Remington, 1992; Evans et al., 1996; Kurz et al., 1998; Mulholland and Richards, 1998).

Glucose transporter 1 (glut1), also known as solute carrier family 2, facilitated glucose transporter member 1 (SLC2A1) is a protein that in humans is encoded by the *SLC2A1* gene (Mueckler et al., 1985)]. Glut-1 is the most ubiquitous glut isoforms present in mammalian cells and tissues (Scheepers et al., 2004; Wood and Trayhurn, 2003) like erythrocytes, placenta, fetal tissues, brain, endothelia and many immortalized cell lines. GLUT1 facilitates the transport of glucose across the plasma membranes of mammalian cells (Olson and Pessin, 1996). Currently, 13 members of the facilitated glucose transporter family (GLUTs) have been identified in mammals (Joost and Thorens, 2001; Zhao and Keating, 2007). Earlier cloning and sequencing study of glut1 cDNA in chicken showed that it shares 95% amino acid sequence similarity to mammalian gluts (Wagstaff et al., 1995).

In our laboratory, heat shock protein HSP70.1 (*HSPA-1A*) was cloned and characterized in buffalo embryos (Sharma et al., 2012). The present study aimed the cloning and characterization of glut 1 and citrate synthase cDNA sequences in buffalo (*B. bubalis*).

MATERIALS AND METHODS

Ooocyte collection and mRNA extraction

Ovaries at random stages of the estrous cycle were collected from local abattoir and transported at 32 to 37°C in 0.9% normal saline to the laboratory within 2 h. Ovaries were rinsed well in pre-warmed phosphate buffer saline (PBS) fortified with antibiotics (75 mg/L penicillin-G, 50 mg/L streptomycin sulphate). Cumulus oocytes complexes (COCs) were collected by aspiration of antral follicles (5 to 8 mm diameter) with 18 gauge needle adapted to 5 ml syringe. COCs (with three to four layer of cumulus) were collected in RNAlater solution (Ambion, Inc, USA) until RNA isolation.

The mRNA was isolated from ninety (90) morphologically good quality oocytes (cumulus oocytes complexes) briefly, cells were lysed in lysis solution and cell lysate was incubated with dynabeads oligo $(dT)_{25}$ for formation of 'Dynabead-mRNA complex'. The 'Dynabead-mRNA complex' was washed twice in 100 µl of each with washing buffer (A) and (B) supplied with the kit and finally eluted in 20 µl of elution volume. For removal of DNA, the eluted mRNA sample was treated with DNAase I as per the manufacturer's protocol (Quigen, GmbH Hilden, Germany). Quality and concentration of isolated mRNA was assessed by Nano-drop (Thermo Scientific, USA).

cDNA synthesis

Reverse transcription was carried out using revert aid kit (Fermentas, Maryland, USA) according to the manufacturer's instructions. Briefly, cDNA was synthesized in a total of 20 µl reaction volume; using 800 ng of RNA template and oligo-dT primers. Reaction was reverse-transcribed using Molony–Murine Leukemia Virus Transcriptase (MMLV-RT) (Fermentas, Maryland, USA) by incubating at 70°C for 5 min followed by incubation at 25°C for 5 min, 42°C for 60 min and finally reaction was stopped by incubating the reaction for 10 min at 70°C. The quality of cDNA was assessed by an amplification reaction for a housekeeping gene β-actin, and amplified product was resolved on 1.5% agrose gel.

Primer designing for RT-PCR

A set of primers for RT-PCR amplification of glut1, citrate synthase and β-actin gene were designed from the conserved coding region of *Bos taurus* sequences. All the primers for RT-PCR were synthesized using primer 3 (v. 0.4.0) software available online through slight adjustment in default parameters set up; quality was checked on primer select software (DNA STAR, USA). Primer sequences, amplicon size, annealing temperature and gene bank accession are listed in Table 1.

Cloning, sequencing, sequence homology and phylogenetic analysis

Primers were synthesized as two primer pairs and single primer pair from bovine (*B. taurus*) complete coding sequences to get amplification of glut1 and citrate synthase respectively. Amplification was carried out in a 50 µl reaction volume containing 10 pmol of each forward and reverse primers, 3 µL template cDNA, 200 µM of dNTPmix, 1.0 mM $MgCl_2$, and 3U proofreading DNA polymerase (MBI, Fermentas, USA) in 1 × Taq buffer. Amplification reaction

Figure 1. Buffalo ovaries and cumulus oocyte complexes (COCs). (A) Buffalo ovaries and (B) Immature oocytes showing 3 to 4 layers of cumulus cells (original magnification of photomicrographs was ×200).

was performed in a thermal cycler (Bioer Technology Co., China) following reaction condition: Initial denaturation at 95°C for 3 min followed by 35 cycles of 45 s at 95°C, at different annealing temperatures (as mentioned in Table 1), extension at 72° C (@1 min/kb), and a final extension of 10 min at 72° C. The amplified products were resolved by agarose gel (1.5 to 2.5%) electrophoresis and visualized over a gel documentation system (Alpha Imager-2200, Alpha Innotech Corporation, Germany) by ethidium bromide (Promega, Madison, USA) staining under UV light.

The amplicons were purified using gel cleanup kit (Advanced microdevices (mdi), Ambala Cant, India) and cloned into pGEMT Easy vector (Promega, Madison, USA), following manufacturer's instructions. Positive recombinant clones were tested for presence of insert by colony and plasmid PCR. Stab culture prepared from three positive clones was sent to Imperial Life Sciences (ILS Bioservices, India) for sequencing. Sequences were subjected to BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) and submitted to gene bank. Nucleotides as well as deduced amino acid sequences were aligned to evaluate their homology with other mammalian sequences using the Clustal W method of MegAlign Programme of Lasergene Software (DNASTAR, USA). Phylogenetic analysis was carried out using MEGA version 4 (Tamura et al., 2007), and buffalo citrate synthase and glut1 structure was predicted using SWISS-MODEL (Arnold et al, 2006).

RESULTS

A total 90 cumulus oocyte complexes collected from 98 buffalo ovaries at 1.12/ovary recovery rate were utilized for RNA isolation (Figure 1). The quality of RNA was assessed by nanodrop (Thermo Scientific, USA) and optical density at 260/280 was found within the acceptable range. Further, the quality of cDNA was assessed by PCR amplification of housekeeping gene, β-actin and a solitary intact band was observed under UV transilluminator (Figure 2E).

Characterization of glut1 and citrate synthase gene

The amplification reaction for complete coding sequence of glut1 and citrate synthase gene was performed using buffalo oocyte RNA (concentration 247.2 ng/µl and OD of 1.906), and final reaction volume 3 µl per 50 µl was used for PCR amplification of gene of interest. Glut1 amplicons of 1091 bp (for primer 1) and 1161 bp (for primer 2) was observed on 1.5% agarose gel and as single amplicon of 1421 bp for citrate synthase which is further confirmed by plasmid PCR (Figures 2A to D). Sequences of complete coding region of glut1 and citrate synthase genes for water buffalo are now available in public domain (Genbank, accession numbers: glut1; HM025989.2, Citrate synthase; JN039302).

Homology and phylogenetic analysis

The nucleotide sequence and predicted amino acid sequences of glut1 and citrate synthase were aligned and compared with available sequences of different mammalian species. Glut1 sequence was compared with cattle (*Bos taurus*), dog (*Cannis lupus familiaris*), horse (*Eqqus caballus*) rabbit (*Oryctolagus cuniculus*), rat (*Rattus norvegicus*) and human (*Homo sapiens*) which revealed the nucleotide substitutions. Buffalo glut1 sequences showed 99% homology with bovine (*B. taurus*), 92% with dog (*C. lupus familiaris*), 92% with horse (*E. caballus*), 92% with human (*H. sapiens*), 89% with rabbit (*O. cuniculus*), and 89% with rat (*R. norvegicus*) and this indicates close evolutionary relationship (Figure 3A). Phylogenetic analysis revealed that *glut1* is a highly conserved gene (89-98% homology among mammalian species) having an open reading

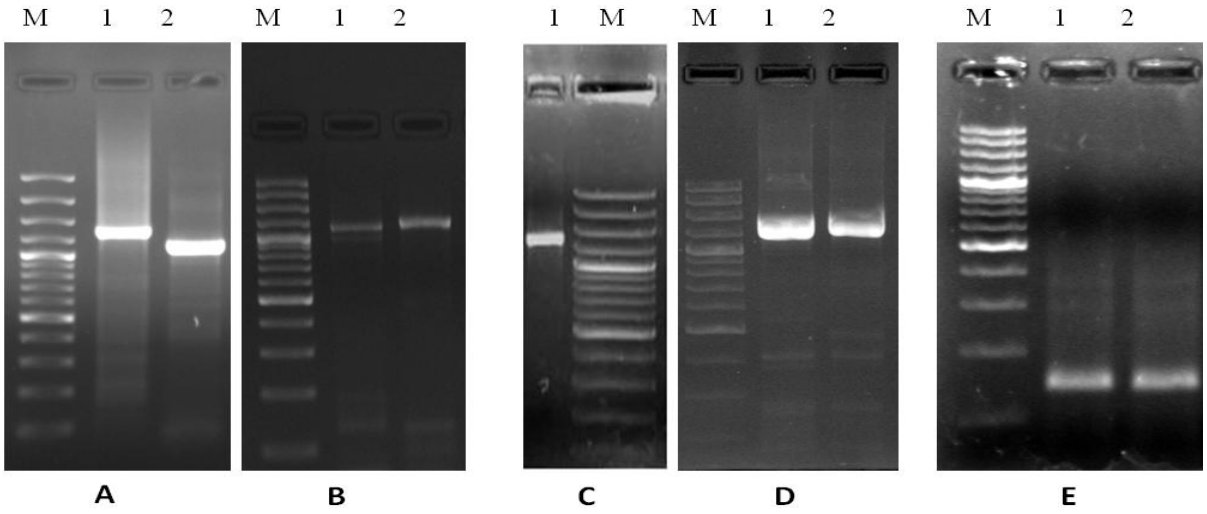


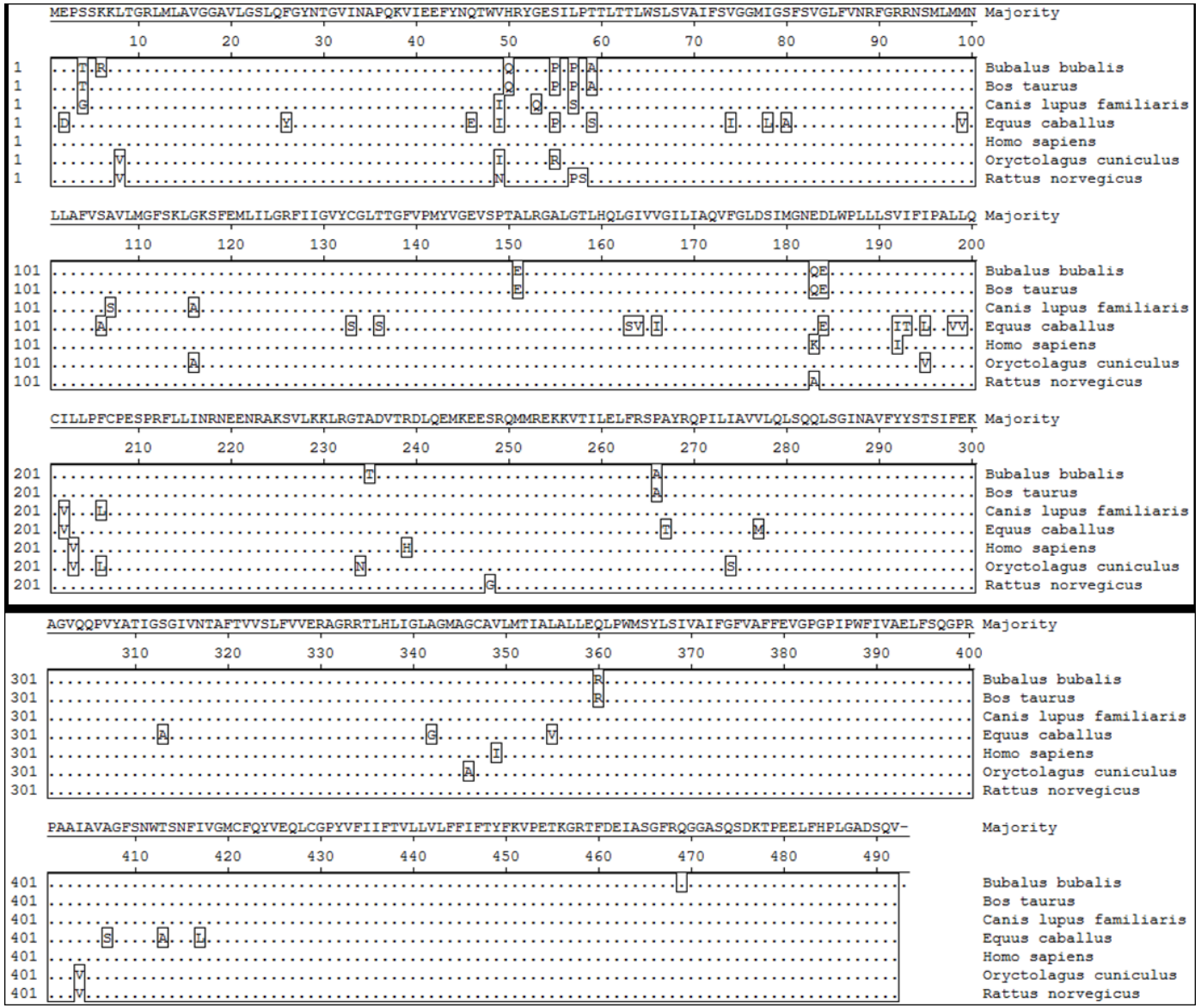
Figure 2. (A) PCR amplification of glut1 complete coding sequences: Lane M, 100bp+ molecular weight marker; Lane 1 (1161 bp) and Lane 2 (1091 bp) amplicons amplified through primer 1 and primer 2 respectively. (**B)** Confirmation of glut1 cloned product through plasmid PCR: Lane 1 (1091 bp) and Lane 2 (1161 bp) for primer 2 and 1, respectively. **(C)** Amplification of Citrate synthase complete coding sequences: Lane M, 100 bp+ molecular weight marker; Lane 1, citate synthase amplicon (1421bp). **(D)** Confirmation of Citrate synthase cloned product through plasmid PCR: Lane M, 100 bp+ molecular weight marker; Lane 1 (1421bp) and Lane 2 (1421 bp) showing plasmid PCR products for citrate synhase. **(E)** Confirmation of cDNA quality through PCR amplification of β-Actin gene: Lane M, 100 bp + molecular weight marker; Lanes 1 and 2, COCs.

frame (ORF) of 1476 bp encoding 492 amino acid. Phylogenetic analysis showed that bovine, rat, mice and humans were derived from different ancestors according to their closer evolutionary relationship (Figure 4A). Among these, cattle and buffalo might have evolved from a common ancestor as expected, pig positioned in between and diverged early from the bovid ancestors, and buffalo *glut1* gene had an individual place, closer to bovine but in different lineage. Further predicted protein structure of buffalo glut1 shows the presence of crucial amino acids for glucose transport (Figure 5A). Similarly, citrate synthase sequences were compared with Cow (*B. taurus*), mice (*Mus musculus*), rabbit (*O. cuniculus*), pig (*Sus scrofa*), rat (*R. norvegicus*) and Human (*H. sapiens*). Buffalo citrate synthase sequences showed ~ 99% homology with bovine (*B. taurus*), 94% with horse (*E. caballus*), 94% with human (*H. sapiens*), 88% with mouse (*M. muscullus)*, 94% with rat (*R. norvegicus*) and 89% with pig (*S. scrofa*) which also shows close evolutionary trend among different domestic specie sand human (Figure 3B). Sequence alignment and phylogenetic analysis shows that citrate synthase is a highly conserved gene (89-99% homology among domestic species) having an open reading frame (ORF) of 1398 bp encoding 466 amino acid. Phylogenetically, bovine, rat, mice and humans were derived from different ancestors according to their closer evolutionary relationship (Figure 4B). Further catalytic amino acid residues in predicted protein structure of buffalo citrate synthase were found conserved (Figure 5B).

DISCUSSION

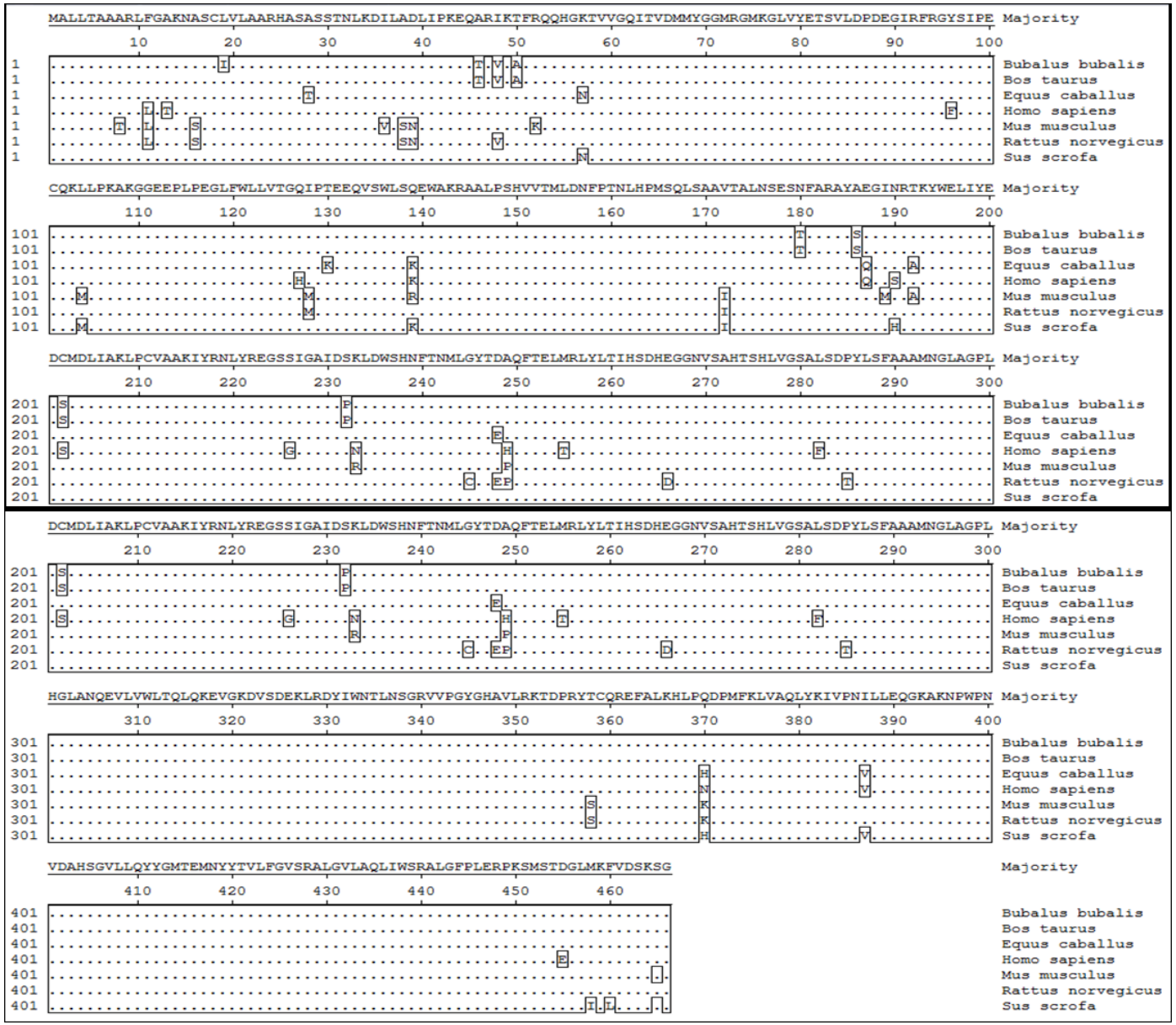
In the present study, we for the first time characterized buffalo cDNA encoding glut1 and citrate synthase genes. The complete coding sequences of buffalo glut1 and citrate synthase genes were analyzed for sequence similarity as well as their phylogenetic relationship amongst other domestic species. Earlier, cDNA encoding the glut1 protein have already been isolated from human, rat, mouse, rabbit, and pig tissues (Mueckler et al., 1985; Birnbaum et al., 1986; Kaestner et al., 1989, Baldwin 1993; Asano 1988) and all exhibits a high level of amino acid identity (97%). Sequence analysis of buffalo glut1 also showed 89 to 98 and 97 to 99% homology at nucleotide and protein level respectively with domestic species and human.

Predicted bubaline glut1 protein has 54060.94 Daltons molecular weight having 491 amino acids; 38 of them are strongly basic (K, R) whereas 32 strongly acidic (D, E), 217 Hydrophobic (A, I, L,F, W, V) and 114 Polar (N, C, Q, S, T, Y) amino acids. Mueckler and colleagues (1985) using hydropathy analysis, predicted that glut1 consists of 12 transmembrane-spanning-helices with the N and C termini and a large loop between transmembrane helices 6 and 7 located on the cytoplasmic side of the membrane (Mueckler et al., 1985, Cairns et al., 1987; Davies et al., 1987; Davies et al., 1990). A smaller loop between transmembrane helices 1 and 2 was predicted to be extracellular (Asano et al., 1991). The bulk of experimental evidence to date supports this model. Cope (1994) also



A

Figure 3. Alignment of predicted amino acid sequence of buffalo (A) *glut1* and (B) *citrate synthase* with different domestic species and human. Identical sequence is indicated by a dot and differences by the corresponding one-letter symbol of the amino acid.



B

Figure 3. Contd.

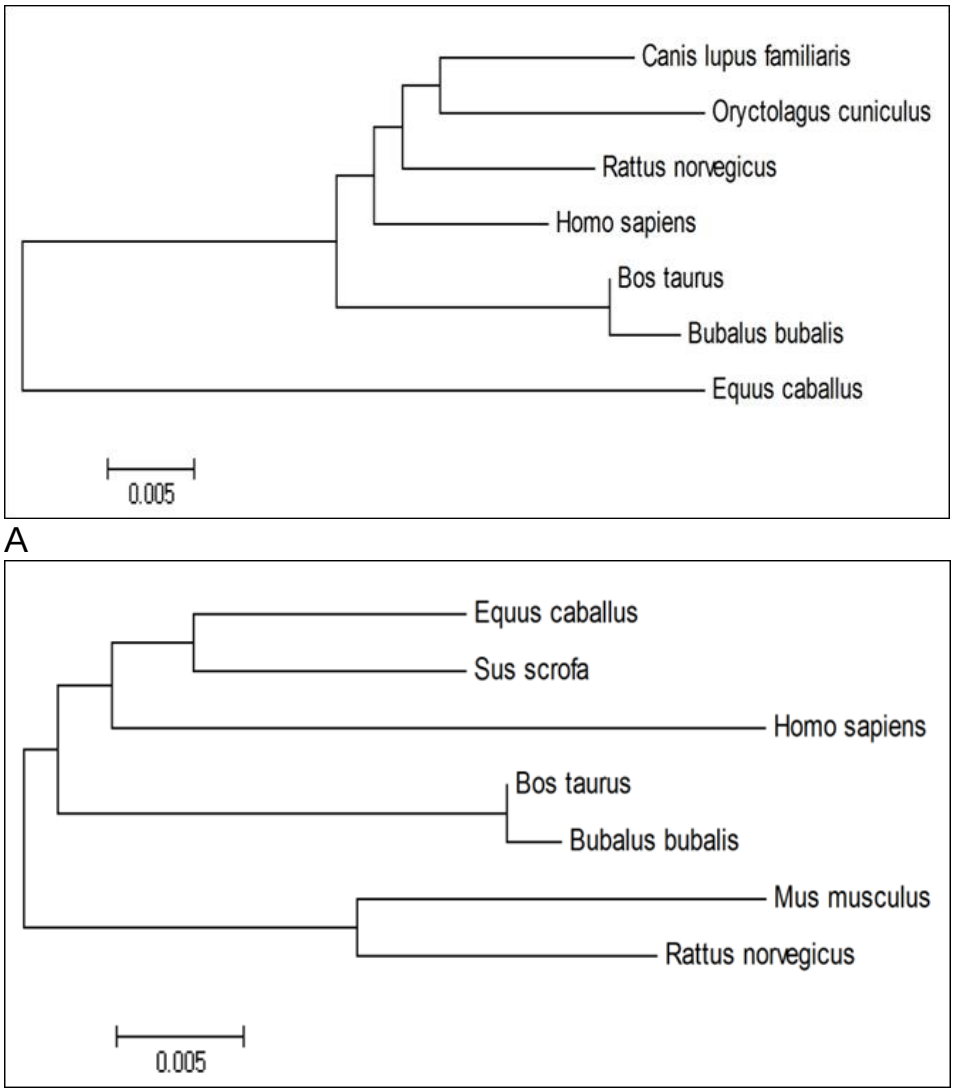


Figure 4. Phylogenetic relationship of (A) *glut1* and (B) *citrate synthase* nucleotide sequences from different species using Mega version 4.1, following the alignment of the ORF sequences using clustal W and neighbour-joining method (nucleotide p distance).

supported the concept of a bilobular structure for the intact glucose transporters in which separate C- and N-domain halves pack together to produce a ligand-binding conformation. Our results of predicted protein structure also support this structure for buffalo glut1. Certain amino acid residues plays critical role in glucose transport. Amongst those G75, G76, G79, N288, and A289 (Olsowski et al., 2000); Q161 (Seatter et al., 1998; Mueckler et al., 1994); V165 (Mueckler and Makepeace, 1997); N317,T321, and P387 (Mueckler and Makepeace, 2002); Q282(Hruz and Mueckler, 1999; Olsowski et al., 2000); I287 (Hruz and Mueckler, 1999); W388 (Kasahara and Kasahara,1998; Garcia et al., 1992); W412 (Garcia et al., 1992) are the crucial amino acids for glucose transport function. These residues were also present in the bubaline predicted structure of glut1 translation.

Sequence homology results for buffalo citrate synthase showed 89 to 99% and 95 to 99% homology at nucleotide and protein level respectively. Earlier citrate synthase was characterized in bovine sequence and was found 92.1 and 93.8% identical to the human and porcine coding sequence, respectively (Winger et al., 2000). The amino acid sequence predicted from the bovine sequence is 95.1% identical to the human sequence and 96.3% identical to the porcine sequence. The porcine sequence contains a stop codon that results in a peptide truncated by 2 amino acids.

Predicted bubaline citrate synthase protein has 51771.66 Daltons molecular weight having 466 amino acids; 47 of them are strongly basic (+) (K, R) whereas 45 are strongly

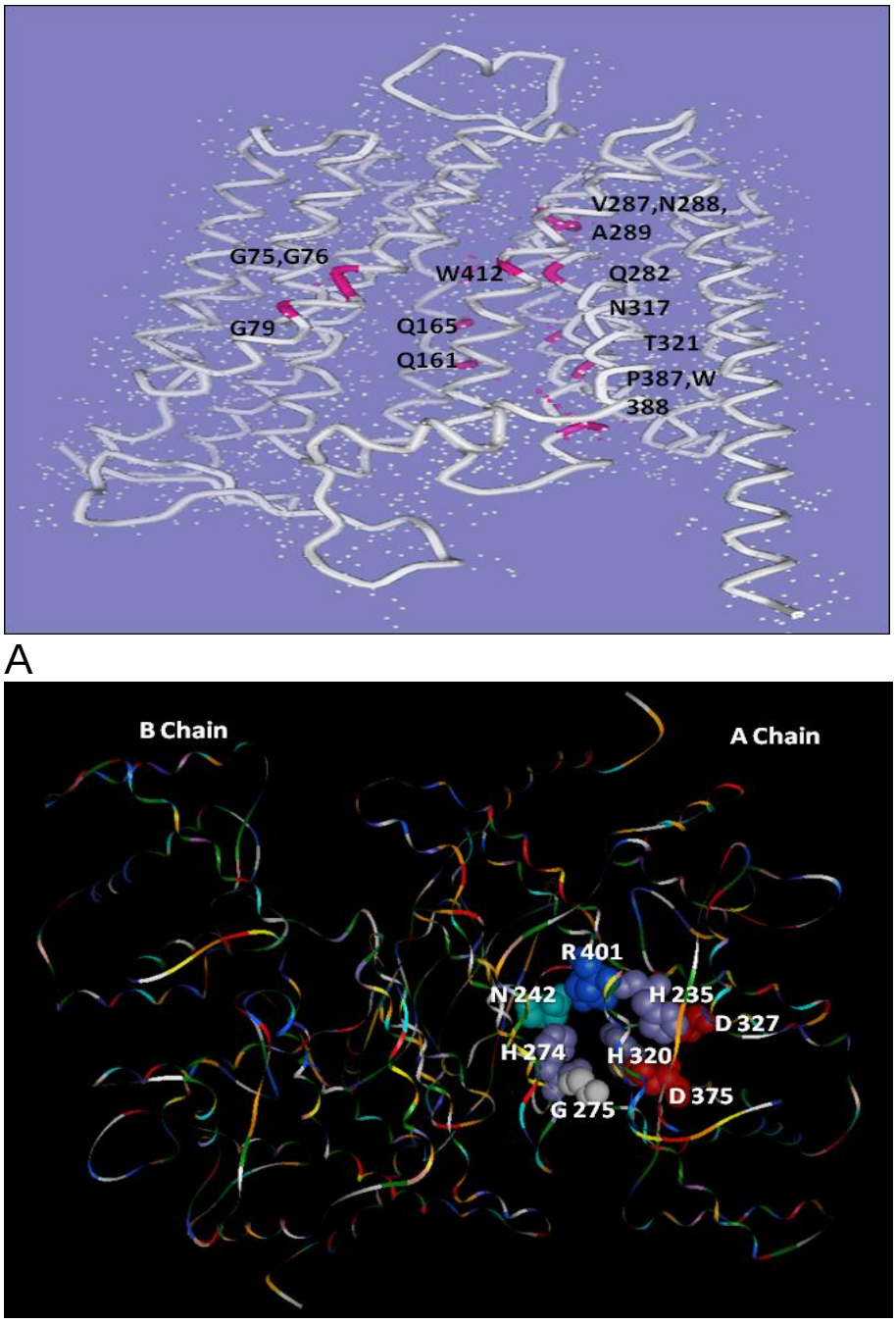


Figure 5. Predicted protein structure of bubaline (A) glut1 and (B) citrate synthase protein (SWISS MODEL).

acidic (-) (D, E), 169 hydrophobic (A, I, L, F, W, V) and 118 Polar (N, C, Q, S, T, Y) amino acids. Predicted structure of buffalo citrate synthase translation showed the presence of highly conserved catalytic residues. Earlier, several studies have been conducted focusing on the same residues, the three catalytic residues (His320, Asp375, His274) and the residues Asp327, Arg401, Gly 275, His235, and Asn242 believed to be involved at the active site or in the conformational change (Evans et al., 1996; Kurz et al., 1998; Mulholland and Richards, 1998). The buffalo amino acid sequences were found conserved with bovine and human amino acid sequences at these eight residues.

This study concludes that *glut1* (89 to 98% homology at

nucleotide and 97 to 99% at amino acid level) and citrate synthase (89 to 99% homology and 95 to 99% at amino acid level) are highly conserved gene among domestic species and human. These sequences were also found conserved in their predicted protein structure for critically active (W412, A289, Q161, V165, N317, T321, P387, Q282, I287, W388, G75, G76, G79 and N288) and catalytic amino acid (His320, Asp375 and His274) residues of buffalo glut1 and citrate synthase, respectively.

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Full Length Research Paper

Contribution towards the development of a DNA barcode reference library for West African mammals

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DNA barcoding is a widely used molecular approach for species cataloging for unambiguous identification and conservation. In the present study, DNA barcoding of some West African mammals were performed with six new mitochondrial CO1 sequences for *Civettictis civetta, Tadarida nigeriae, Orycteropus afer, Heliosciurus gambianus, Equus africanus asinus* and *Funisciurus anerythrus* which are absent in public databases such as BLAST/NCBI and BOLD. Sequence identifications were made by comparing unknown sequences against the DNA barcodes of known species through distance-based tree construction and alignment probing. The sequences have been deposited to GenBank/NCBI.

Key words: mtDNA, West African mammals, conservation, biodiversity.

INTRODUCTION

West Africa is endowed with rich flora, fauna and variety of ecosystems. These ecosystems contain wide variety of endemic species with highly restricted ranges within the region. In Nigeria, for instance, there are 290 mammals of which one species is critically endangered, 13 are endangered, 16 are vulnerable, and 10 are near-threatened (IUCN, 2012). However, many species present in this diverse geographic area have not been classified and this has hampered progress in understanding species richness and for developing strategies for conservation efforts (Burgess-Herbert et al*.,* 2010). The nomenclature of organisms is essential for bridging taxonomical research between other disciplines of science catering for human welfare. However, this process to categorize biological diversity is greatly affected by the lack of taxonomic expertise (Ke and Loren, 2006).

Since its initial proposal as a tool for rapid identification of species, a technique using a primer set to amplify a 648-base pair (bp) region of the mitochondrial cyto-chrome-*c* oxidase subunit 1 (*COI*) gene to ensure rapid and accurate identification of a broad range of biological specimens (Hebert et al*.,* 2003), DNA barcoding has gained considerable validation. Among terrestrial verte-brates, this approach has been shown to be effective in the identification of amphibians (Smith et al*.,* 2008), North American birds (Hebert et al., 2004; Kerr et al*.*, 2007) Neotropical birds (Kerr et al*.,* 2009) and Neotropical small mammals (Clare et al., 2007). The basic idea behind DNA barcoding is the comparison of nucleotide sequence of a standard gene region of an unknown species with a reference library of known DNA barcode to establish a species identity for the query. Ergo, DNA barcoding cannot be used for species identification when there are no barcode records for the query sequence in the reference library (Wong et al*.,* 2011). Development of a reference DNA barcode library of all the known species identified by taxonomists are the first and most important step to establish DNA barcoding as a tool for accurate species identification. Due to the lack of a DNA

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Table 1. List of mammal species used in the present study.

West African mammals species studied	Collection locality	GenBank accession number for CO1
Carnivora: Viverrinae		
Civettictis civetta	Otuocha	JX426124
Felidae		
Felis catus	Orba	JX426133
Chiroptera: Molossidae		
Tadarida nigeriae	Obimo	JX426125
Artiodactyla: Bovidae		
Tragelaphus scriptus	Uvuru	JX426130
Bos taurus	Nru	JX426135
Rodentia: Sciuridae		
Heliosciurus gambianus	Uvuru	JX426127, JX426128
Funisciurus anerythrus	Uvuru	JX426129
Muridae		
Rattus rattus	Nsukka	JX426131
Tubulidentata: Orycteropodidae		
Orycteropus afer	Uvuru	JX426126
Perissodactyla: Equidae		
Equus ferus caballus	Obollo afor	JX426134
Equus africanus asinus	Obollo afor	JX426132

reference library for the West African mammals, the latter have scarcely been identifiable (Burgess-Herbert et al., 2010).

Biodiversity conservation in the fields of ecology, evolutionary biology, agriculture, economics among others relies on accurate species identification, which is pivotal to the basic aspect of recognizing and describing biodiversity. For instance, unlike the usual classification based on morphological aspects according to taxonomic studies involving ingenuity of taxonomists and trained technicians to identify taxa accurately with accumulation of special skills over the years through experience is the use of molecular instead of morphological data for identifying taxa, which has long been a fundamental idea of many biologists (Busse et al., 1996; Blaxter 2003). Progressive achievements in DNA-sequencing technologies have enabled researchers studying biodiversity to conduct simple, cost-effective and rapid DNA analyses. This progress in biotechnology, and the taxonomy crisis itself, played a large role in the creation of DNA barcoding (Jinbo et. al., 2011).

A good number of taxonomists are alarmed that DNA barcoding will compete with the age long traditional taxonomic studies for example Ebach and Holdrege (2005a, b). However, DNA barcoding is inseparably linked to taxonomy, a potent tool that compliments taxonomic studies (Schindel and Miller 2005; Hajibabaei et al., 2007). The integration of various types of data, such as morphological, ecological, physiological and molecular data, including DNA barcodes, will improve species finding and description practices (Waugh, 2007; Padial et al., 2010). This synergic approach will be supported by various biodiversity databases (Jinbo et. al., 2011). Nevertheless, Judging from its wider recognition now as a veritable identification tool, DNA barcoding has become a very important type specimens description in the developed parts of the world unlike parts of the world like Africa. The present work was designed in such a way to generate DNA barcode sequences of some of the West African mammals, which may enhance building a reference DNA barcode library of Western Africa.

MATERIALS AND METHODS

Eleven (11) mammalian species (Table 1) collected from various parts of West Africa were selected for the study after confirming their taxonomy. DNA was extracted from alcohol preserved muscle tissue (~25 mg) by using Qiagen DNeasy Blood and Tissue kit. Universal primers were used in the present study for amplifying CO1 gene (Ivanova et al., 2007). Polymerase chain reactions (PCRs) were performed in 25 µl reactions consisting of 2.5 µl each of 10x PCR buffer, $MgCl_2$ (25 mM) and 0.5 µl dNTPs (2 mM), 0.25 µl of each primer (10 µM), 1 µl of Taq DNA polymerase, 14 µl of dH_2O and 4 µl of template DNA (10-20 ng) in a Thermocycler (ABI 9700). The following thermo cycling conditions were used for amplifications: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 52°C for 40 s, 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were visualized on 1% agarose gels and the most intense products were purified using Exo Sap IT (USB). Bidirectional sequencing was performed using the PCR primers and products were labeled with BigDye Terminator V.3.1 Cycle sequencing Kit (Applied Biosystems, Inc.) and sequenced in an ABI 3730 capillary sequencer following manufacturer's instructions. The sequences were aligned using ClustalW and potentially misaligned sequences were excluded. The

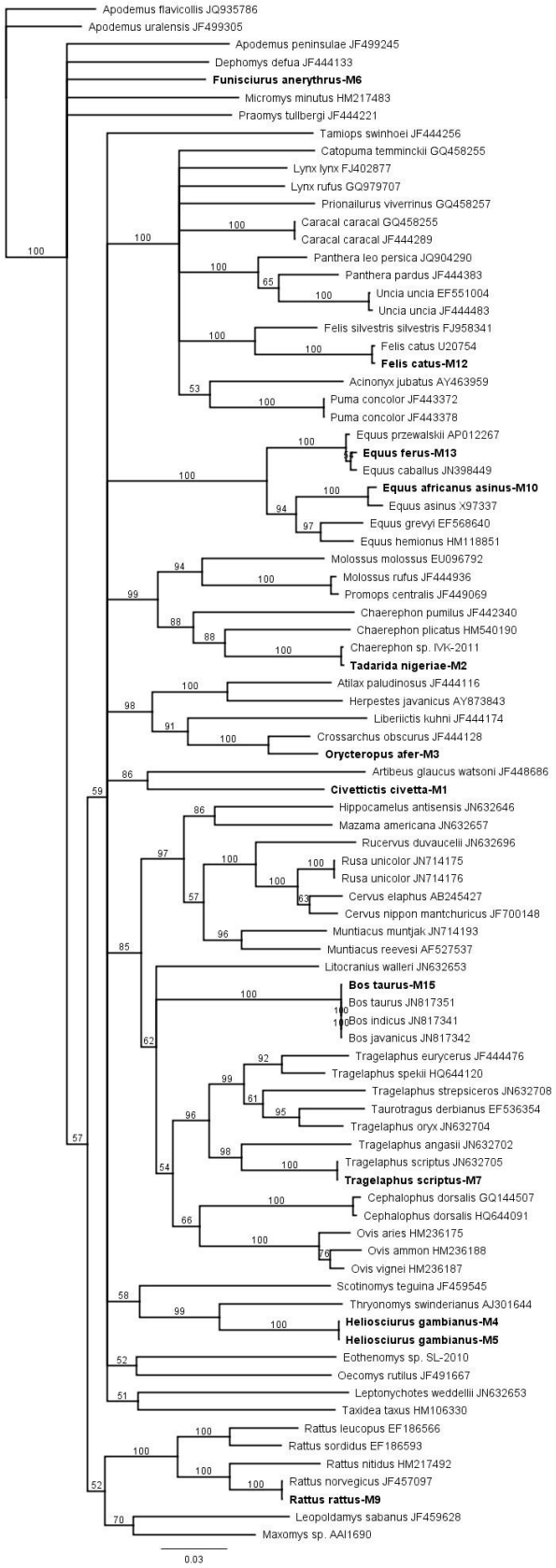

Figure 1. Neighbour joining tree based on *CO1* gene (500 bootstrap replicates).

extent of sequence differences between species was calculated averaging pair-wise comparisons of sequence differences across all individuals. Pairwise evolutionary distance was determined by the Kimura-2-parameter method using the software programme Mega 5 (Tamura et al*.,* 2011). The number of polymorphic sites and nucleotide diversity (pi), nucleotide composition and number of transition and transversion between species were determined. Gaps were considered as missing data on the phylogenetic reconstructions. Neighbour Joining (NJ) tree was constructed to show intraspecific and interspecific relationships among the new sequences and related sequences in GenBank. The new sequences are deposited in GenBank. (Accession numbers are given in Table 1).

RESULTS AND DISCUSSION

All samples were successfully sequenced for CO1 using the forward and reverse primers to obtain robust forward and reverse sequences of approximately 582 bp. No insertions, deletions or stop codons were observed in any sequence of CO1. After alignment, there were 336 common sites in the partial sequence of CO1 genes including inserted gaps used for this analysis. No areas of uncertain alignment were identified. After filtering, there were 246 (42.2 %) variable sites and all of them were phylogenetically distinct. Based on analyses of the estimates of evolutionary divergence between sequences, the nucleotide composition was extremely guanine poor T = 30.1%, A= 27.3%, C = 26.5% and G = 16.2% with 42.7% GC content. The overall mean divergence (d) of the studied samples was 0.227 (Table 2), conserved sites 356, variable sites 226, parsimony informative sites 192 and singleton sites 34. The sequence comparison of CO1 sequence data of West African mammals revealed that six among the 11 species included in the present study were not represented earlier in public data bases (BOLD and GenBank). They are *Civettictis civetta, Tadarida nigeriae, Orycteropus afer, Heliosciurus gambianus, Equus africanus asinus* and *Funisciurus anerythrus.* Neighbour joining (NJ) tree based on CO1 sequence generated in the present study as well as the sequences of all the West African mammals available with the GenBank are represented in Figure 1.

C. civetta (Schreber, 1776) is commonly known as African civet and is listed as least concern as the species has a wide distribution range with a variety of habitats and present in many protected areas. It may be undergoing some localized declines due to hunting and might be rendered more vulnerable in areas where preferred bush meat becomes scarce (IUCN, 2012). *T. nigeriae* (Thomas, 1913) is commonly known as Nigerian Free-tailed Bat feeding primarily on arthropod species. There are 106 species of bat reported from West Africa including fruit bats and insectivorous bats (Okafor et al*.,* 2004). Many of the latter are house dwelling while all of the former are predominantly found in the wild. The aardvark *O. afer* (Pallas, 1766) is a medium-sized,

Table 2. Estimates of evolutionary divergence between sequences.

Species	1	2	3	4	5	6	7	8	9	10	11	12
Civettictis civetta-M1	0.000											
Equus africanus asinus-M10	0.249	0.000										
Felis catus-M12	0.226	0.287	0.000									
Equus ferus-M13	0.234	0.100	0.266	0.000								
Bos taurus-M15	0.253	0.214	0.302	0.229	0.000							
Tadarida nigeriae-M2	0.221	0.223	0.246	0.199	0.235	0.000						
Orycteropus afer-M3	0.194	0.231	0.241	0.256	0.220	0.215	0.000					
Heliosciurus gambianus-M4	0.239	0.229	0.278	0.232	0.227	0.245	0.238	0.000				
Heliosciurus gambianus-M5	0.239	0.229	0.278	0.232	0.227	0.245	0.238	0.000	0.000			
Funisciurus anerythrus-M6	0.240	0.260	0.285	0.240	0.209	0.236	0.221	0.205	0.205	0.000		
Tragelaphus scriptus-M7	0.258	0.248	0.281	0.240	0.163	0.229	0.185	0.204	0.204	0.216	0.000	
Rattus rattus-M9	0.228	0.262	0.250	0.239	0.221	0.227	0.206	0.214	0.214	0.163	0.241	0.000

burrowing, nocturnal mammal native to Africa. It is the only living species of the order Tubulidentata although other prehistoric species and genera of Tubulidentata are known (IUCN, 2012). *E. africanus asinus* (Linnaeus, 1758) commonly known as African Ass is a domesticated member of the Equidae or horse family. Small numbers of donkeys are kept for breeding or as pets in developed countries (IUCN, 2012). *H. gambianus* (Ogilby, 1835) is commonly known as Gambian sun squirrel and believed to be a complex of several similar species (IUCN, 2012). This species is typically associated with savanna woodlands. Populations have also been observed within riparian forest and in savanna areas. It is generally absent from closed forest habitats. This species is commonly found in agricultural areas, especially oil palm plantations. Animals are diurnal, solitary and predominantly arboreal. The sequence obtained in the present study may serve as a reference material for further research to address the question of complex species status of this group. *F. anerythrus (*Thomas, 1890) is commonly known as Redness Tree Squirrel or Thomas's Rope Squirrel. This species is generally found in lowland tropical moist forest throughout much of the range, but has also been reported from gallery forest. It can be found in secondary habitats. Animals are usually found singly or in pairs (IUCN, 2012).

The West African population present in Benin and Nigeria might represent a distinct species for which the sequence obtained in the present study may help further taxonomic studies needed to resolve this question. Limitations in the available specimens and molecular data have prevented us from covering major portion of the West African mammal species in this study. It is also necessary to continue with studies focused on mammals throughout their distribution with the additional aim of investigating the phylogenetic and taxonomic status in various habitats. This will help in proposing conservation policies for species associated with these habitats in West Africa. Further molecular works and their cross references with morphological and ecological studies will provide new insights into the phylogeny and taxonomy of West African mammals.

Ethical considerations

All the ethical issues (including prevailing laws on the collection of samples from the wild in Nigeria) have been completely observed by the authors.

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Full Length Research Paper

Genetic diversity and structure of potamodromous *Opsaridium microlepis* (Günther) populations in the inlet rivers of Lake Malawi

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Studies were carried out to determine the genetic diversity and structure of endangered *Opsaridium microlepis* (Mpasa) populations in the affluent rivers of Lake Malawi; Linthipe, Bua, Dwangwa and North Rukuru. A total of 200 DNA samples of *O. microlepis* from four river populations were analyzed at 20 microsatellite loci. The primers' discriminating power was high (mean PIC, 0.76) yielding a total of 295 alleles with a range of 10-22 and an average of 15 alleles per locus. All the populations were not in Hardy Weinberg Equilibrium probably due to outbreeding that leads to heterozygosity excess. This observation was further supported by heterozygosity excess exhibited by 100% of the population-locus combinations (mean $F_{IS,}$ -0.30) and lack of evidence for genetic bottleneck. The populations exhibited high genetic diversity as evidenced by high mean Shannon information Index (I=1.64) and high observed heterozygosity (H_o = 0.98). Genetic relationships among the populations appear to be less influenced by geographical distance (Mantel's statistics Z, 0.18; *p* = 0.6369) implying that the populations do not fit into the isolation by distance model. Nevertheless, the populations are highly differentiated (F_{ST} = 0.17; AMOVA among populations = 16%). This is supported by inter-deme migration of less than one individual per generation (*Nm*=0.91) as determined by Slatkin' private allele method. Therefore, these populations are probably still large and distinct requiring separate monitoring and management due to inferred restricted gene flow and considerable population differentiation.

Key words: Mpasa, Lake Malawi, population structure, genetic diversity, microsatellites, threatened species, conservation.

INTRODUCTION

The genetic diversity and population structuring of native species populations is an important feature to be considered in conservation and management programs. The use of molecular techniques to assess patterns of genetic variation has supported conservation programs, by indicating which species require greater conservation efforts and also by selecting areas where natural populations are viable (Johnson et al., 2001). Recently, molecular markers have been commonly used in population studies. Simple sequence repeat (SSR) markers are preferable because they are co dominant, highly polymorphic and are considered the most powerful in terms of their resolving power (Creste et al., 2004; Buhariwalla et al., 2005). In addition, microsatellites have a wide distribution in the

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genome and can be easily identified, making them ideal markers for genetic variability studies of populations (Collevatti et al., 2001). The polymorphism obtained with microsatellite markers has provided useful information necessary for management of fish stocks (Alam and Islam, 2005), population analysis and biodiversity conservation (Romana-Eguia et al., 2004; Changadeya et al., 2012).

The fisheries production from the wild is generally declining and with an increase in human population, the pressure is even greater on the resource (Government of Malawi, 1998). Most of the fish species in Lake Malawi including cichlids and cyprinids are endemic. Studies by Ambali et al. (2002) have also shown that there are species flocks that are endemic to specific areas in the lake. Pollution, lakeshore development and other human activities have degraded critical habitats for the spawning and early life history stages of many fishes. In addition, recruitment, overfishing and reduction of spawning stock below a critical threshold have prevented many populations from rebuilding to previous levels of abundance. Riverine potamodromous cyprinids species are most vulnerable because they are targeted by fishermen during the spawning period when they swim up stream. This development has led to the fishery of some in-let rivers of Lake Malawi such as North Rukuru, Bua, Dwangwa and Linthipe to be threatened with extinction. Though there are several commercially important riverine cyprinids such as Mpasa (*Opsaridium microlepis*) that are under threat, they have been studied to a limited extent resulting in inadequate knowledge of their biogeography, origin and genetic diversity (Snoeks, 2004).

Mpasa, belong to the Cyprinidae family and is endemic to Lake Malawi. This species was described by Günther in 1864, and is mostly caught while on spawning runs in large rivers (Lowe, 1975; Tweddle, 1981). In recent years, *O. microlepis* catches in Lake Malawi and its in-let rivers have declined (Anon, 2008). The inclusion of Mpasa in the 2006 IUCN Red List of Threatened species (IUCN, 2006) was because it had been heavily fished during its spawning runs up rivers and was considered to have consequently suffered approximately 50% decline in the past ten years, due to overfishing, habitat degradation and pollution (Kazembe et al., 2005). This is a cause for concern in the conservation of Mpasa and development of fisheries in Malawi in general. Therefore, the objective of this study was to investigate genetic diversity and population structuring among populations of *O. microlepis* in the Lake Malawi' river in-lets of Bua, Dwangwa, Linthipe and North Rukuru using microsatellite markers.

MATERIALS AND METHODS

Sample collection

Muscles tissues of 200 *O. microlepis* specimens (50 each from North Rukuru, Bua, Dwangwa and Linthipe river) (Figure 1) were collected in February and April 2010. Tissue of about 5-10 mm^2 was extracted from individual fish and preserved in vials with 95% ethanol. The samples were brought to Molecular Biology and Ecology Research Unit DNA Laboratory, Department of Biological Sciences, Chancellor College, University of Malawi, where DNA analysis was conducted using 20 microsatellite markers.

DNA extraction and amplification

Total genomic DNA was extracted from *O. microlepis* samples following a standard SDS- proteinase K/phenol-chloroform protocol as described by Hillis et al. (1996). The study used 20 polymorphic microsatellite loci (Table 1). Amplification of DNA was done in 13.11 µL final reaction volume with 5.7 µl PCR grade water, 1 µl of 10 mM dNTP mix, 1.25 µl of 10 x PCR buffer, 1.6 µl of 25 mM magnesium chloride ($MgCl_2$), 0.75 µl of 15 pmol of both forward and reverse primers, 0.06 µl of 5u/µl *Taq* DNA Polymerase stored in buffer A (Promega, USA) and 2 µl of 25ng/ µl template DNA. The amplifications were carried out in a Master-cycler gradient 5331 Eppendorf Version 2.30.31-09 with the following PCR conditions: initial denaturation at 94°C for 2 min, then 10 amplification cycles of denaturing at 94°C for 30 s, annealing at an optimal temperature for a specific primer pair for 15 s and elongation at 72°C for 30 s. This was followed by 30 amplification cycles of denaturing at 89°C for 30 s, annealing at an optimal temperature for a specific primer pair for 15 s and elongation at 72°C for 30 s. The final extension was at 72°C for 20 min followed by a soaking temperature of 4°C.

Electrophoresis of PCR products on polyacrylamide gel using silver staining technique

The amplified PCR products were resolved using 6% polyacrylamide gel electrophoresis. The 6% polyacrylamide gel was poured in BIORAD Sequi-Gen® GT Nucleic Acid Electrophoresis Cell. A total of 6 µL of STR 3X Loading Solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added to the PCR products in 0.2 mL PCR tubes and denatured in Mastercycler gradient at 95°C for 5 min. Then 6 µL of denatured PCR products was loaded on the 6% polyacrylamide gel and ran at 50 W. The gel plates were fixed, stained and developed following procedures in the Promega Silver Sequence™ DNA Sequencing System Technical Manual. The microsatellites bands were scored over a light box using pGem DNA marker (Promega, USA) and ϕ X174 DNA/*Hinf* 1 (Promega, (USA) as band size standard markers.

Data analysis

Polymorphism information content (PIC), a variability measure of each locus, was calculated as described by Zhao et al. (2007):

$$PIC = 1 - \left(\sum_{i=1}^{n} p_i^2 \right) - \sum_{j-1}^{k-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2$$

Where, p_i is the frequency of the ith allele out of the total number of alleles at a microsatellite locus, and n is the total number of different alleles for that locus.

Genepop on the web (Raymond and Rousset, 1995) was used to conduct the following analyses: test for conformity to Hardy-Weinberg Equilibrium (Haldane, 1954; Weir, 1990; Guo and Thompson, 1992), test for genotyping linkage equilibrium using Fisher' method, tests of genic and genotypic differentiation, estimation of effective number of migrants using Slatikin's private allele method (Slatkin, 1985) and computation of Wrights statistics (Wright, 1969).

Bottleneck computer software version 1.2.02 (Cornuet and

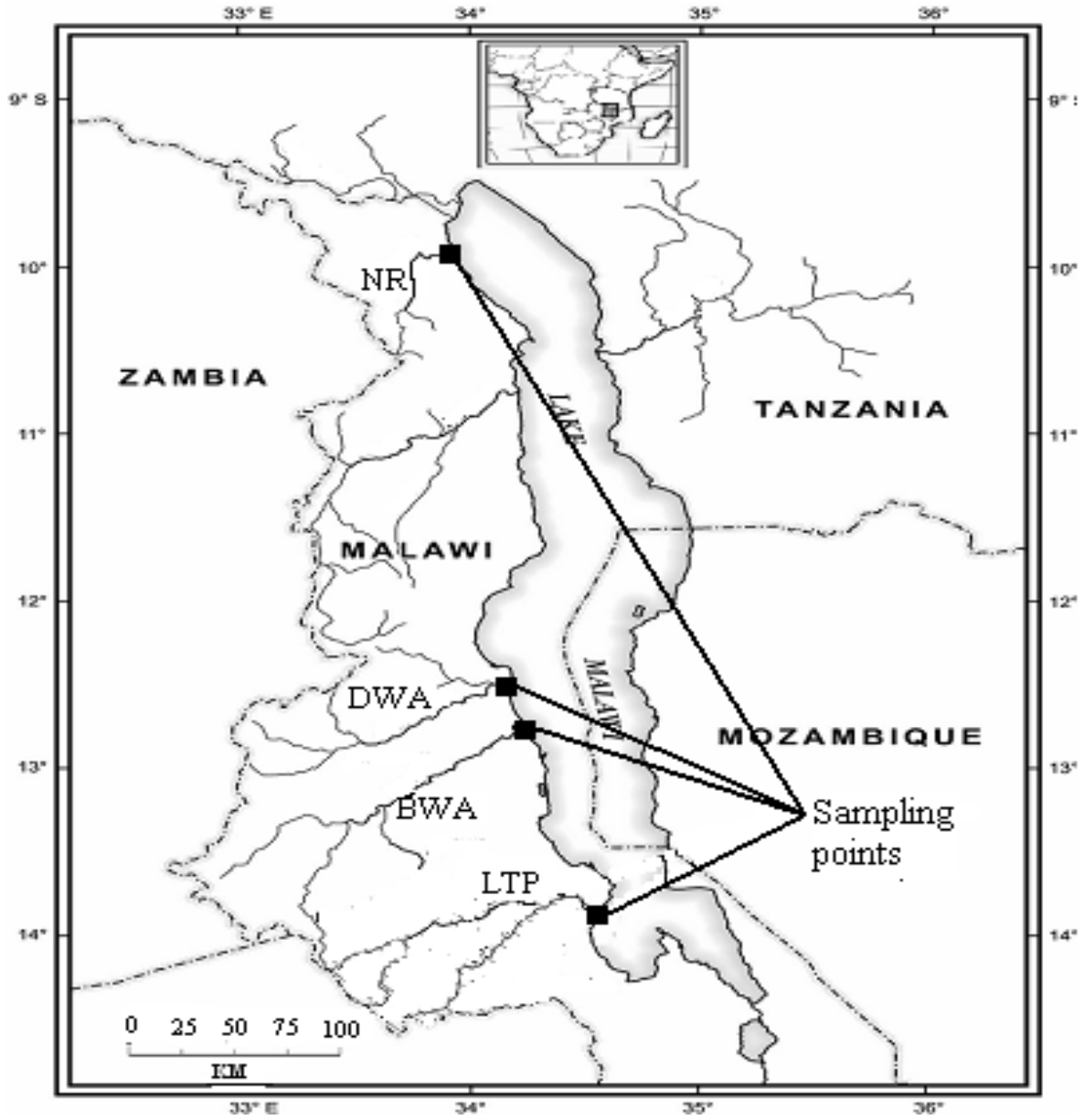

Figure 1. Rivers from which *O. microlepis* populations were sampled. NR, North Rukuru River; BWA, Bua River; DWA, Dwangwa River; LTP; Linthipe River (Source: www.map of the world.com).

Table 1. Total number of alleles (A) and allele size range (SR) in base pairs and microsatellite primer polymorphic information content (PIC).

Locus	A	Gene Bank accession	T_A	Allele size range (SR)	Repeat motif	Primer source (reference)	PIC
CypG49	12	AY349167	53.6	170-192	$(TA)_{11-21}$	Baerwald and May (2004)	0.74
CypG3	11	AY349122	56.4	150-202	$(CAGA)_2$	Baerwald and May (2004)	0.76
CypG13	13	AY349132	53.4	140-172	$(TAGA)_{10}$	Baerwald and May (2004)	0.73
Ca3	10	AF277575	54.5	162-182		Dimsoski et al., (2000) cited by Hamilton and Tyler (2008)	0.78
CypG5	12	AY349124	54.3	114-172	$(TAGA)_{12}$	Baerwald and May (2004)	0.76
CypG4	17	AY349123	54.3	134-202	$(TAGA)_{12}$	Baerwald and May (2004)	0.79
CypG30	22	AY349148	54.3	118-182	$(TAGA)_7$	Baerwald and May (2004)	0.77

Table 1. Contd.

Lid1	17		55.3	140-194	(TTTC)7	Barinova et al., (2004) cited by Hamilton and Tyler (2008)	0.75
CypG48	16	AY349166	55.3	126-158		Baerwald and May (2004)	0.80
MFW	11	EF144124	55.3	106-126		Mohindra et al., (2005)	0.76
CypG22	12	AY349140	55.7	202-224		Baerwald and May (2004)	0.70
CypG6	12	AY349125	52.5	192-218	$(TAGA)_7$	Baerwald and May (2004)	0.71
CypG8	18	AY349127	52.5	132-166	$(CAGA)_6$	Baerwald and May (2004)	0.73
CypG21	14	AY349139	52.7	158-184	$(CAGA)_6$ $(TAGA)_7$	Baerwald and May (2004)	0.75
CypG27	14	AY349145	52.7	104-188	$(TAGA)_8$	Baerwald and May (2004)	0.76
Lid11	16		53.7	200-228	(TTTG)8	Barinova et al., (2004) cited by Hamilton and Tyler (2008)	0.77
AP1	13	AJ428582	53.4	158-182	$(TA)_{11-21}$	Hsu et al.,(2004)	0.74
AP2	18	AJ428583	55.0	110-188	$(AC)_{18-20}$	Hsu et al.,(2004)	0.78
Ru2	17		53.6	142-174		Barinova et al., (2004) cited by Hamilton and Tyler (2008)	0.75
CypG15	20	AY349134	53.8	116-158		Baerwald and May (2004)	0.82
Mean	14.75						0.76

T_A, annealing temperature.

Luikart, 1996) was employed to determine if the populations in the study had undergone recent effective population size reductions (Genetic bottlenecks). The bottleneck tests estimations were based on 1000 replications using three mutation models; Infinite Allele Model (IAM), Stepwise Mutation Model (SMM) and Two-Phased Model (TPM). Three statistical tests were employed namely; Sign test, Standardised Differences Test and Wilcoxon Sign Rank Test.

POPGENE Version 1.31 freeware (Yeh et al., 1999) was used to compute several measures of genetic variability within and between sample populations. The following variables were computed: Shannon's information index (I) (Lewontin, 1974), genetic distance and other population variance measurements such as observed heterozygosity (Ho) and expected heterozygosity (He).

Shannon' Information Index means for the four populations were compared at 95% level of significance using unpaired *t* test with Welch's correction performed by Graph Pad Prism version 3.00 (1999), for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com.

NTSYSpc version 2.11c (Rholf, 2001) used genetic distances matrices for the four populations generated by POPGENE Version 1.31 to construct a genetic relationships dendrogram from the Sequential Agglomerative Hierarchical and Nested (SAHN) clustering method using the Unweighted Pair-Group Method with Arithmetical averages (UPGMA) algorithm (Sneath and Sokal, 1973).

Mantel's test was undertaken to determine correlations between genetic and geographical distance matrices among the populations. The MXCOMP programme of NTSYS was used to compute a product-moment correlation coefficient (normalized mantel's statistics Z) for the two matrices (Rholf, 2001). In order to determine if the correlation was significant, actual coefficient was compared with the values produced by randomly permuting the matrix pair 2000 times.

The significance of the spatial variation in gene diversity among populations was estimated by performing a hierarchical analysis of genetic diversity among populations using the analysis of molecular variance model (AMOVA), as described in Michalakis and Excoffier (1996) using ARLEQUIN version 3.1 (Excoffier et al., 2006).

RESULTS

Genetic diversity

All 20 loci were polymorphic in all populations with polymorphic information content (PIC) values averaging 0.76 with a range of 0.70 at locus CypG22 to 0.82 at locus CypG15 (Table 1). This implies that all SSR loci used in this study had high discriminating power since PIC ≥ 0.5 is indicative of high polymorphism locus (Botstein et al., 1980). A composite genotypic linkage disequilibrium analysis for each locus pair across all four populations at 20 loci, showed that 19 (10%) locus pairs out of 190 possible locus pairs were in significant linkage disequilibrium (data not shown). This shows that genotypes at 90% of all loci were not linked. A total of 295 alleles with a mean of 15 per locus and a range of 10 to 22 alleles were generated reflecting a rich allelic diversity in the populations. A mean Shannon Information Index (I) of 1.64 was generated and showed that the populations exhibited high genetic diversity.

All populations deviated significantly from Hard-Weinberg Equilibrium (HWE) at all loci ($p \leq 0.05$) (Table 2). The mean observed heterozygosity (H_o) was higher than expected heterozygosity (H_e) averaging 0.98 and 0.87 and ranging from 0.92 to 1.00 and from 0.78 to 0.92, respectively (Table 2). The Wright's Fixation index (F_{IS}),

Table 2. Summary of estimates of observed and expected homozygosity and heterozygosity, Wright' F-Statistics and results of exact test of Hardy Weinberg Equilibrium (HWE) at 20 loci for all populations.

Locus	HWE	H_o	H_e	Obs. Hom.	Exp. Hom.	F_{IS}	F_{IT}	F_{ST}
CypG49	0.0000	0.98	0.86	0.02	0.14	-0.32	-0.09	0.18
CypG3	0.0000	0.92	0.78	0.08	0.22	-0.27	-0.15	0.10
CypG13	0.0000	1.00	0.81	0.01	0.19	-0.35	-0.18	0.12
Ca3	0.0000	0.93	0.83	0.07	0.17	-0.18	-0.10	0.06
CypG5	0.0000	0.97	0.79	0.03	0.21	-0.28	-0.22	0.05
CypG4	0.0000	0.96	0.85	0.04	0.15	-0.27	-0.10	0.14
CypG30	0.0004	0.96	0.90	0.04	0.10	-0.32	-0.01	0.23
Lid1	0.0000	0.96	0.90	0.04	0.10	-0.27	-0.02	0.19
CypG48	0.0000	0.97	0.91	0.03	0.09	-0.23	-0.03	0.16
MFW	0.0000	1.00	0.87	0.00	0.14	-0.31	-0.12	0.15
CypG22	0.0000	1.00	0.86	0.00	0.14	-0.44	-0.09	0.24
CypG6	0.0000	1.00	0.86	0.00	0.14	-0.40	-0.10	0.22
CypG8	0.0000	1.00	0.90	0.00	0.10	-0.37	-0.05	0.23
CypG21	0.0000	1.00	0.90	0.00	0.14	-0.30	-0.13	0.13
CypG27	0.0000	1.00	0.90	0.00	0.14	-0.31	-0.12	0.14
Lid11	0.0000	1.00	0.91	0.00	0.09	-0.31	-0.04	0.21
AP1	0.0000	1.00	0.86	0.00	0.14	-0.34	-0.11	0.17
AP2	0.0000	1.00	0.90	0.02	0.11	-0.26	-0.06	0.16
Ru2	0.0000	1.00	0.90	0.01	0.10	-0.32	-0.05	0.20
CypG15	0.0001	0.97	0.92	0.03	0.08	-0.25	-0.01	0.19
Mean	0.0000	0.98	0.87	0.02	0.13	-0.30	-0.09	0.17

H_o, Observed heterozygosity; H_e expected heterozygosity; obs. Hom. observed homozygosity; Exp. Hom. expected homozygosity; Wright' F-Statistics (F_{IS}, F_{IT} & F_{ST})

values were negative across all loci in all populations indicating an excess of heterozygotes (heterozygotes excess), in contrast to the positive values of F_{IS} which indicate excess of homozygotes (heterozygotes deficiency) (Table 2).

Bottleneck tests revealed that all the populations had a normal L-shaped allele frequency distribution as expected under mutation drift equilibrium, suggesting that the populations had not experienced a recent genetic bottleneck (data not shown).

Genetic structure and differentiation among populations

Tests for a genic and genotype differentiation showed that all population pairs were significantly differentiated ($p \leq 0.05$) (Table 3). Similar results were reflected by mean Shannon Information Index which showed that all the population pairs were significantly different except LTP-DWA pair ($p \leq 0.05$) (Table 3). The estimate of population differentiation (F_{ST}) among the population pairs ranged from 0.14 to 0.19 with BWA-LTP pair being the least differentiated and LTP-DWA and NR-DWA pairs the most differentiated (Table 3). The overall differentiation among the populations was 17% (F_{ST}=0.17) (Table 2). The 17% level of differentiation was indicative of high genetic differentiation and was validated by Analysis of Molecular Variation (AMOVA) which showed that among population variation was at 16.4% (Table 4). Population differentiations of more than 15% are considered high rather than moderate (Wright, 1978; Hart and Clark 1997) and are associated with low gene flow among the populations. The study registered an overall low gene flow of less than one migrant per generation among the four populations (Nm=0.91). The highest number of migrants per generation (N*m* = 0.70) was observed between LTP and BWA populations and the lowest gene flow was between NR and DWA populations (Nm=0.38) (Table 3).

Cluster analysis and genetic relationships among populations

Cluster analysis dendrogram indicated that BWA and LTP populations were the most genetically close and DWA population was genetically isolated from the rest of the populations (Figure 2). Although BWA and LTP populations were genetically close, but geographically, Bua River is close to Dwangwa River (Table 3). Mantel's test revealed weak insignificant positive correlation between genetic and geographical distances among the populations (r = 0.18; p=0.6369). Thus, the structuring in *O. microlepis* populations is not necessarily due to isolation

Table 3. Summary of contingency tests of differentiation (Fisher' exact Probability test), Nei's (1972) genetic distance, gene flow (Nm), Population Fixation Index (F_{ST}), geographical distance and Shannon Information Index (I) between population pairs.

Population pair	Parameter							
	Genic and genotypic Differentiation	Genetic distance	Nm	F_{ST}	Geographical distance (km)	Shannon Information Index (I)		
	P -values					Mean±SD	Mean±SD	*P-values*
NRand BWA	0.0000	1.05	0.67	0.16	335.9	1.66±0.14	1.73±0.16	0.008***
NR and LTP	0.0000	0.82	0.49	0.16	451.9	1.66±0.14	1.58±0.15	0.006***
BWAand LTP	0.0000	0.74	0.70	0.14	122.11	1.73±0.16	1.58±0.15	0.0001***
NR and DWA	0.0000	1.24	0.38	0.19	294.24	1.66±0.14	1.58±0.13	0.0001***
BWAand DWA	0.0000	0.90	0.56	0.16	43.98	1.73±0.16	1.58±0.13	0.0001***
LTP and DWA	0.0000	1.06	0.45	0.19	158.85	1.58±0.15	1.58±0.13	0.8201ns

NR, North Rukuru River; BWA, Bua River; DWA, Dwangwa River; LTP; Linthipe River.

Table 4. Analysis of molecular variation (AMOVA) among and within population of *O. microlepis* (based on 1023 permutation and over 20000 bootstraps).

Source of variation	Sum of squares	Variance components	Percentage Variation
Among Population	458.83	1.48	16.40
Within Population	2931.2	7.54	83.60
Total	3390.02	9.02	-

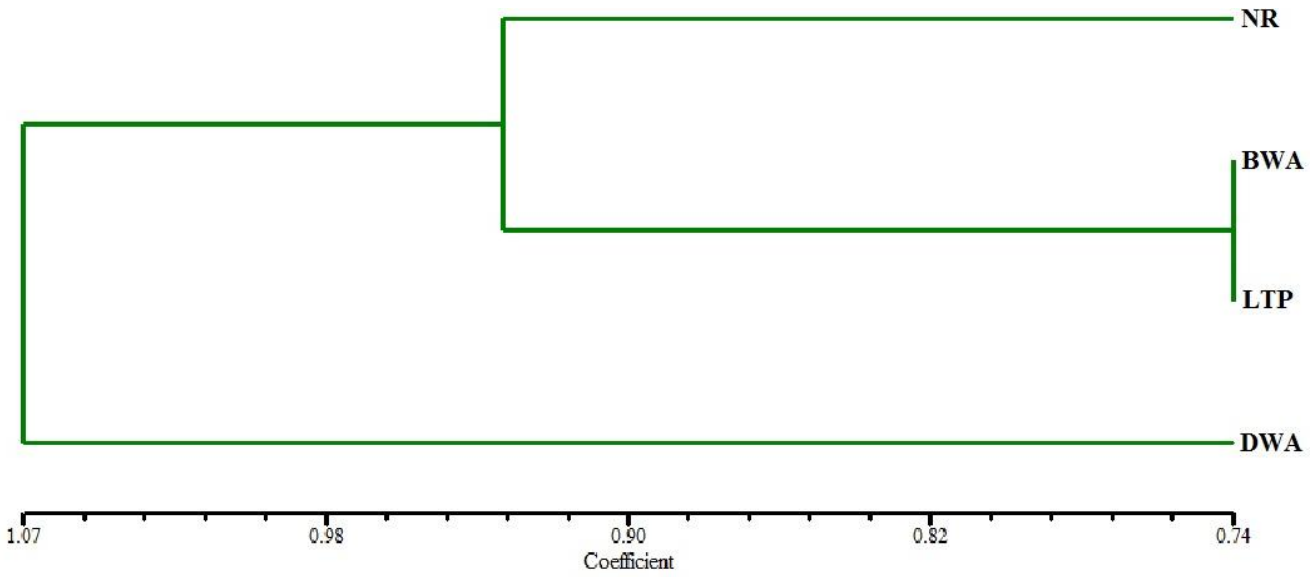

Figure 2. Genetic relationships among the populations based on Nei' genetic distances.

by geographical distances.

DISCUSSION

Genetic diversity

Maintenance of genetic diversity is a major component of many species conservation programs, since loss or critical reduction of genetic diversity is one indicator of diminution in evolutionary potential. In this study, Mpasa, an endangered species due to declining numbers caused by overfishing, demonstrated high degree of genetic diversity, based on total allele count of 295 resulting in a high average number of alleles per locus of 15. This observation was further supported by high mean observed heterozygosity (0.98) and high mean Shannon Information Index (1.64). The mean values of number of alleles per locus and observed heterozygosity reported in this

study are higher than those found in other cyprinid species that have experienced a reduction in historic population size but still retain high genetic diversity, such as the Cape Fear shiner (A = 8.2, H_e = 0.70) reported by Saillant et al. (2004); *Anaecypris hispanica* (A = 10.3, H_e = 0.68) reported by Salgueiro et al. (2003) and the critically endangered cyprinid, *Squalius aradensis* (A=4.9, H_e=0.45) reported by Mesquita et al. (2005). The observed phenomenon (high genetic diversity after presumed reduction in population size) can generally occur when a species has recently experienced a modest decline in population size which has not been sustained over many generations. Continued reductions of even modest proportions, can lead to serious decrease in genetic diversity (Frankham et al., 1995) so continuous monitoring is recommended. However, bottleneck tests results in this study cast some doubts on the presumed reduction in population size among the four Mpasa populations, since they reveal that the populations have not experienced recent reduction in effective population size. Therefore, the observed high genetic diversity could be due to out breeding within the populations.

Conformity to Hardy-Weinberg Equilibrium and test for linkage disequilibrium

Determination of whether a fish stock is a mixture of fish from more than one population is commonly done using a test of Hardy-Weinberg Equilibrium and a test for linkage disequilibrium. Both tests are based on the genetics principle that a mixture of gene pools will exhibit a Wahlund effect, that is, homozygote excess relative to binomial distribution (Kamonrat, 1996). All the populations of *O. microlepis* were not in Hardy-Weinberg Equilibrium at all loci and had negative values for F_{IS} (Table 2) at all loci suggesting that an excess of heterozygotes was responsible for the departure from HWE (Rosewich et al., 1999). The heterozygosity excess could be due to out breeding of the populations since contrary to popular expectations; bottleneck tests have shown that the *O. microlepis* populations in the study have not experienced a recent genetic bottleneck despite known over exploitation over the years. However, Peery et al. (2012) argued through a review of published literature that typically applied, microsatellite-based bottleneck tests often do not detect bottlenecks in populations of vertebrates known to have experienced declines. Their simulations revealed that bottleneck tests displayed limited statistical power to detect bottlenecks due to limited samples sizes and loci used in reviewed published studies (median=8-9 loci and 31-38 individuals). If bottleneck tests limitations are applied to this study, then excess of heterozygotes, which imply occurrence of recent genetic bottlenecks, probably would be due to small number of breeders producing the next generation leading to allelic frequencies in males and females parents differing due to binomial sampling error (Pudovkin et al., 1996; Stoeckel et al., 2006). This difference in allele frequencies in males and females results in an excess as regards HWE of heterozygotes in progeny hence populations deviating from HWE (Pudovkin et al., 1996). Further, Balloux (2004) argued that in small sexual or self-incompatible populations, the fact that individuals cannot reproduce with themselves decrease the probability of creating homozygote offspring.

The study registered 10% of loci pair wise linkage disequilibrium at 5% level indicating a possible admixture though at a lower level.

Genetic structure and differentiation within and among population

AMOVA results revealed that 84% of genetic variation resides within populations indicating possibly, the existence of large enough populations that allow reasonable random mating. The 16% among population genetic variance implies great genetic differentiation among the populations as validated by F_{ST} value of 0.17. Wright (1978) considered any F_{ST} value above 0.15 as showing high genetic differentiation. Level of genetic differentiation demonstrated by Mpasa in this study (Table 2) is higher as compared to values seen in Pacific herring (F_{ST} = 0.023), Atlantic herring (F_{ST} = 0.035) and widespread anadromous fish like Atlantic salmon (F_{ST} = 0.054) (McConnell et al., 1995). Low gene flow among populations (Nm=0.91) and absence of recent genetic bottlenecks which implies that despite heavy exploitation, the populations are outbreed due to possible presence of large numbers of fish, are the plausible reasons for the observed high genetic differentiation among the populations. The high F_{ST} obtained in this study hence signify that the populations are distinct requiring independent conservation management for each river system. Mills and Allendorf (1996) concluded that rate of migration of $Nm \geq 1$ leads to considerable homogeneity among populations but population divergence and structuring occurs when $Nm \leq 1$. The populations in the present study have overall migration rate of $Nm \leq 1$ rendering them to structuring and divergence.

Genetic relationships and cluster analysis of the populations

Although comparison of Shannon Information Index population means found that Linthipe and Dwangwa population pair was insignificantly different (p=0.8201), Wright F statistics (F_{ST} = 0.19) revealed that the pair was among the most differentiated with second lowest gene flow (Table 3). Bua and Linthipe population pair though not the closest geographically is the most genetically close (Figure 2) possibly due to common founding population which is reflected in form of more shared alleles (highest gene flow) among the population pairs resulting in the least genetic differentiation (Table 3).

The low relationship between genetic and geographical distance as revealed by Mantel's test denotes that the populations do not fit into the isolation by distance model. This model states that gene flow is the highest between close populations and it is expected that close populations should show similar genetic composition but it is not the case with these populations. Findings of this study concur with other studies of *Lenthrinops* species flock (Duponchelle et al., 1999; Changadeya et al., 2001) which reported fish flocks not fitting the isolation by distance model though in those studies the fish populations experienced high migration rates.

Conclusions

The results showed that populations in the study are probably still large, have high genetic variation and are highly differentiated as to be considered as distinct populations. Therefore, the four inlet river populations (North Rukuru, Bua, Linthipe and Dwangwa) require separate monitoring and management strategies due to inferred restricted gene flow and population differentiation.

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Full Length Research Paper

Biosynthesis of silver nanoparticles by *Leishmania tropica*

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Biosynthesis and characterizations of nanoparticles have become an important branch of nanotechnology. A novel biosynthesis route for Silver Nanoparticles (Ag-NPs) was attempted in the present study using *Leishmania tropica* the causative agent of cutaneous leishmaniasis in different countries, particularly in Mediterranean region in Iraq. Silver nanoparticles were successfully synthesized from $AgNO_3$ by reduction of aqueous Ag+ ions with the cell of *L. tropica*. AgNPs were irregular spherical in shape and the average particle size was about 35±5 nm characterized by means of UV–vis absorption spectroscopy and scanninng electron microscopy (SEM) images. The efficiency of *L. tropica* for synthesis of silver nanoparticles was found to be higher; also this method was cost effective and easily scaled up for large scale synthesis.

Key words: *Leishmania tropica*, biosynthesis, silver, nanoparticles.

INTRODUCTION

Leishmaniasis is a parasitic diseases induced by 20 different species of *Leishmania.* Leishmaniasis is reported from 88 countries and estimated that 350 million worldwide are at risk of acquiring one form of the diseases, and 12 million are infected with annual incidence rate of about 1.5 to 2 million. According to WHO estimates, 90% of cutaneous cases occur in six countries. Leishmaniasis depends upon causative agent and host genetic background presents various manifestations ranging from a self healing lesion to a lethal systemic form of the disease. Two most common clinical forms of the disease Cutaneous Leishmaniasis (CL) and visceral leishmaniasis (VL) are mainly seen in 14 of the 22 countries of Eastern Mediterranean Region (EMRO) (Postigo, 2010). Traditionally, *Leishmania* parasites are directly detected by microscopic examination of clinical specimens. However, in an endemic area, CL can generally be diagnosed by its clinical appearance alone (Kaur et al., 2003). Nanotechnology is the study of controlling matter on an atomic and molecular scale. Generally, it deals with structures of the size of 100 nm or smaller in at least one dimension, and involves developing materials or devices within that size (Kim et al., 2010). Biological methods of nanoparticles synthesis using microorganisms (Klaus et al., 1999; Konishi and Uruga, 2007), enzymes (Willner et al., 2006), fungus (Vigneshwaran et al., 2007), and plants or plant extracts (Shankar et al., 2004; Ahmad et al., 2011) have been suggested as possible eco-friendly alternatives to chemical and physical methods. The development of green processes for the synthesis of nanoparticles is evolving into an important branch of nanotechnology especially silver nanoparticles, which have many applications (Armendariz et al., 2002; Kim et al., 2010; Kyriacou et al., 2004). Chemical synthesis methods lead to presence of some toxic chemical absorbed on the surface that may have adverse effect in the medical applications.

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Green synthesis provides advancement over chemical and physical method as it is cost effective, environment friendly, easily scaled up for large scale synthesis and in this method there is no need to use high pressure, energy, temperature and toxic chemicals (Singh et al., 2010; Jain et al., 2009).

In recent years, silver nanoparticles (Ag-NPs) have attracted considerable attention for medical and chemical applications due to their exceptional properties including antibacterial activity, high resistance to oxidation and high thermal conductivity (Feng et al., 2006; Lee, 2007; Soni and Prakash, 2011). Recently, findings have demonstrated that nanosilver has anti-inflammatory effects and increases wound healing and dressings of wounds. If these results are confirmed *in vivo*, nanosilver may be appropriate for ulcer treatment (Karla et al., 2010).

The aim of the present study was to evaluate the synthesis of silver nanoparticles using *Leishmania tropica* and determine the characteristics of produced material. This study is the first record of biosynthesis of silver nanoparticles by *L*. *tropica* in Iraq.

MATERIALS AND METHODS

Samples collection

Forty four (44) patients were selected for isolation of *Leishmania* species from their cultures. Skin scrapings from the lesion were obtained and smears prepared on a slide, stained with Giemsa and examined microscopically for presence of amastigotes. Bacterial contamination of *Leishmania* cultures was minimized by cleaning lesions with 70% methanol and local debridement before obtaining specimens. At least two Giemsa-stained slides for each patient were prepared for microscopic examination and cultured.

Culture

The samples were aspirated from the edge of the skin lesions and cultured in liquid phase (normal saline) of Novy MacNeal Nicolle (NNN) media. The culture was incubated at 25°C and checked for growth of *Leishmania* promastigotes for 28 days. Penicillin-G and streptomycin were added to the phosphate buffered saline (PBS) solution utilized in the NNN media culture (Eisenberger and Jaffe, 1999; Farahmand et al., 2008).

PCR

PCR assay was performed according to the manufacturer's protocol (Sinagen, Iran) with the final volume of 25 µL of each PCR reaction. PCR amplification was carried out in a DNA Thermal Cycler (Master cycler gradient, San Leonardo, Canada) based on the following conditions: initial denaturation (95°C, 3 min; 63°C, 30 s; 72°C, 60 s) 1 cycle followed by 35 cycles including denaturation (93°C, 20 s), annealing (63°C, 20 s) and extension (72°C, 40 s). Finally, 10 µL of amplified samples without adding loading buffer were loaded in a 2% agarose gel containing 0.5 mg/ml ethidium bromide in electrophoresis and the products were visualized by ultraviolet (UV) transillumination.

Synthesis of Ag-NPs

The cell culture of *L. tropica* was kept under stirring (5,000 rpm) (5 g) for 5 min at 28°C till it were separated from the broth culture then the settled cells were washed with distilled water three times. One gram (1 g) of wet cell mass was then resuspended separately in 0.001 M $AgNO_3$ solution at pH 5.4 to 6.0. The total mixture was left in room temperature (25°C) for 3 h, and the reaction carried out. For characterization, nanoparticle powders were diluted in pure acetone and the prepared suspension was ultrasonicated. The biotransformation was routinely monitored by visual observation of the biomass as well as measurement of the UV–Vis spectra from the leishmanial cells. The positive reaction appear as formation of a yellow- brown color of the medium due to the reduction of silver ions and production of silver nanoparticles in the medium (Figure 1). The scanning electron microscopy used SEM grids were prepared by taking small amount of sample powder on a copper coated grid and dried under lamp. The silver nanoparticles appeared as spherical in shape and the average size was from 35 to 40 nm with inter-particle distance.

RESULTS AND DISCUSSION

Diagnosis of *L. tropica* by PCR, culture and microscope

44 patients were detected for *Leishmania* amastigotes by microscopic observation out of which, 38 (86.4%) were positive; however, the NNN culture led to the growth of promastigotes in 40 samples (90.1%). Also, the results shows that all of the 10 samples were positive (100%) from the PCR assay (Figure 2).

UV–vis spectroscopy and SEM

L. *tropica* cells when exposed to silver ions showed a distinct and fairly broad UV–Vis absorption band centered at 425 nm because this nanoparticle was well dispersed without aggregation. The appearance of this band, which was assigned to a surface plasmon, is well documented for various metal nanoparticles with sizes ranging from 2 to 100 nm (El-Raheem et al., 2011). SEM showed the formation of silver nanoparticles with an average size of 35 to 40 nm with inter-particle distance (Figure 3). The shapes of Ag-NPs proved to be spherical. These results confirm the presence of primary and secondary amines bonds; C=O, N=O, C=N and COOH bonds of proteins as capping and stabilizing agent on the nanoparticles surface (Fatemeh and Bahram, 2012; Ramezani et al., 2012).

Development of reliable and eco-friendly process for the synthesis of metallic nanoparticles is an important step in the field of application of nanotechnology. Chemical synthesis methods lead to presence of some toxic chemical absorbed on the surface that may have adverse effect in the medical applications. Biosynthesis provides advancement over chemical and physical method as it is cost effective, environment friendly, easily scaled up for large scale synthesis and in this method there is no need to use high pressure, energy, temperature and toxic chemicals. Therefore, there is most need of silver nanoparticles synthesized by biological methods of plant extract instead of other toxic methods (Singh et al., 2010;

a b

Figure 1. Color change results from adding $AgNO_3$ to the Leishmanial cells; a- before reaction and b- after reaction time of 3 h.

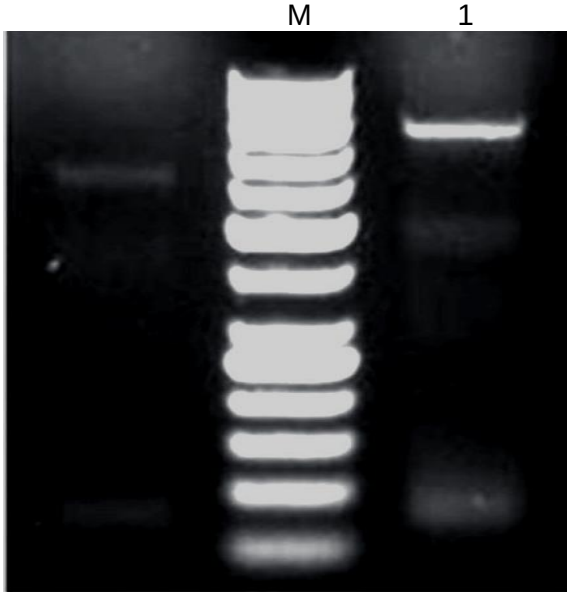

Figure 2. Electrophoretic patterns of PCR products obtained from crude parasite genomic DNAs for *Leishmania* species detection 1, *L. tropica*; M. marker.

Jain et al., 2009; Vyom et al., 2009). The present study confirmed that *L. tropica* is capable of producing silver nanoparticles within 3 h in contrast to other researchers who found that the main problem in the biological nanoparticles is the slow rate of production (Narayanan and Sakthivel, 2010). This approach towards synthesis of silver nanoparticles has many advantages such as process scaling up, economic viability and safe way to produce nanoparticles. Parasitic diseases (like malaria, leishmaniasis, trypanosomiasis) are major health problems around the globe (Edward and Krishna, 2004). Antiparasitic chemotherapy is the only choice of treatment of these parasitic infections. The reason for this is that these infections do not elicit pronounce immune response hence effective vaccination may not be possible (Watkins, 2003).

The main reason for using Ag-NPs in this study was their capacity to produce reactive oxygen species (ROS), which *Leishmania* parasites are known to be susceptible to. Recent studies showed that their wide surface areas, small sizes, and their ability to bind sulfur- and phosphorus-containing groups may lead to an increase in their antileishmanial effects. It was observed that Ag-NPs decreased the metabolic activity and proliferation values of parasites compared with the control groups (Allahverdiyev et al., 2011). This inorganic nanoparticle has a distinct advantage over conventional chemical antimicrobial agents. The most important problem caused by the chemical antimicrobial agents is multidrug resistance. Therefore, an alternative way to overcome the drug resistance of various microorganisms is needed desperately, especially in medical devices, etc. Ag ions have been used for decades as antimicrobial agent in various fields because of their growth-inhibitory capacity against microorganisms (Kim et al., 2007). Our results are in agreement with other results in different parts of the world (Allahverdiyev et al., 2011; Singh et al., 2010; Kim et al., 2007), and disagrees with others (Narayanan et al., 2010; Sondi and Salopek-Sondi, 2004). The nanoparticles were primarily characterized by UV-vis spectroscopy, which was proved to be a very useful technique for the analysis of nanoparticles.

The reduction silver ions and formation of stable nano-

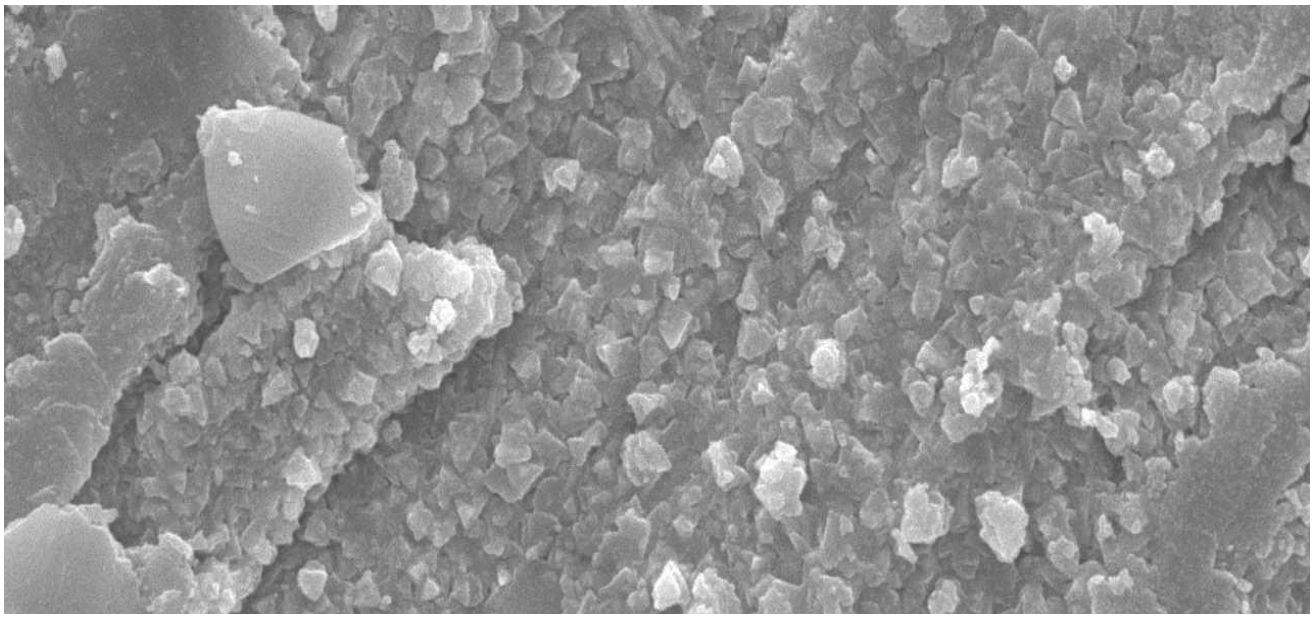

Figure 3. Silver nanoparticles recorded by scanning electron microscopy (SEM).

particles appeared quickly within 3 h of reaction making it one of the fastest bioreducing methods to produce silver nanoparticles (Kim et al., 2007). Reduction of silver ions present in the aqueous solution of silver complex during the reaction with the cell culture of *L. tropica* observed by the UV-Vis spectroscopy revealed the presence of silver nanoparticles may be correlated with the UV-Vis spectra. UV-Vis spectroscopy is well known to investigate the shape and size of nanoparticles. The scanning electron microscopy analysis confirmed the bioreduction of Ag+ ions to silver nanoparticles as well as morphological dimensions of Ag-NPs demonstrated that between 35 to 40 nm with inter-particle distance and they have irregular spherical shape. These findings are in agreement with those of other researchers (Fatemeh and Bahram, 2012; Ramezani et al., 2012; Ponarulselvam et al., 2012). This study concluded that the efficiency of *L. tropica* for synthesis of silver nanoparticles was found to be higher; also this method was cost effective and easily scaled up for large scale synthesis.

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Full Length Research Paper

Effects of seed fermentation method on seed germination and vigor in the oleaginous gourd *Lagenaria siceraria* (Molina) Standl.

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Supplying high quality seed to rural farmers is the basic requirement for the sustainable development of agriculture in developing countries. The present study was conducted to examine the influence of *Lagenaria siceraria* seed fermentation method on seed germination and vigor. Three seed fermentation methods (fermented in ambient air, plastic bag stored in ambient or in plastic bag buried) were tested on two cultivars during two years. Seed germination and vigor were better when fermentation was conducted in anaerobic, darkness, and at low temperature. Low seed quality was observed in unfermented seed, suggesting the occurrence of postharvest maturity in *L. siceraria*. Seed quality did not vary between cultivars. Regardless of the fermentation process and cultivars used, the best seed and seedling qualities were observed when the amount of rainfall during the experiment period was high, suggesting that wet soil is necessary for an on farm reliable evaluation of seed fermentation method in the bottle gourd *L. siceraria*.

Key words: Cucurbit, *egussi*, maturity, minor crops, oilseed, seedling, viability.

INTRODUCTION

The seeds of the oleaginous *Lagenaria siceraria* are consumed as cake or thickeners of a traditional dish called *egussi* soup in most countries from Western and Central Africa (Bisognin, 2002; Enujiugha and Ayodele-Oni, 2003). This cucurbit is reported to be rich in nutrients (Badifu, 1993), namely protein (36±2.17% of dry content (DC) and fat (45.89±4.73% DC). In addition, it is commonly found in many traditional cropping systems, and it is well adapted to extremely divergent agro-ecosystems and various cropping systems characterized by minimal inputs (Zoro Bi et al., 2005). *L. siceraria* thus represents an excellent plant model for which improved cropping systems implementation can insure the economic prosperity of rural people from tropical Africa where the main

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Abbreviations: SFA, Seeds fermented by exposing the cut fruits in ambient air in the field; **SFB,** seeds fermented by packing the cut fruits into plastic bag that was exposed in ambient air in the field; **SFD,** seeds fermented by packing the cut fruits into a plastic bag that were buried at 30 cm depth; **GSI,** germination speed index; **ESI,** emergence speed index; **GeP,** germination percentage; **EmP,** emergence percentage; **SSL,** seedling shoot length; **SDB,** seedlings dry biomass; **RFC,** round-fruited cultivar.

producers are women.

In spite of *L*. *siceraria* nutritional and agronomic potentials, in depth investigations on the crop are scanty (Williams and Haq, 2002). As for most orphan crops, one of the main constraints the breeders must overcome to ensure a sustainable production is the development of improved seed supply systems (Almekinders et al., 1994). The sustainable production of any crop depends mainly on its seed quality. Indeed, uneven seed germination in a field, particularly in direct-seeded crops can lead to variable density and lack of uniformity in plant growth and phenology (Nerson and Paris, 1988). This may have a negative impact on yield and market value of the crop. In most cucurbit seed production systems, seed fermentation in the juicy endocarp and mesocarp tissues of the fruits (Modgil et al., 2004) is the first step after fruit cutting is employed for various days depending on the species. Seed extraction procedure requires care for seed production in the indigenous cucurbits (Nerson, 2007).

Seeds of *L*. *siceraria* are extracted after a 7-day fermentation period of cut fruit containing a juicy pulp to which they are attached (Zoro Bi et al., 2003). This process is expected to facilitate the seed separation from the surrounding tissues, and increase their nutritive value and germination percentage (Achinewhu and Ryley, 1986). Diverse microorganisms are involved in cucurbits seed fermentation process (Harper and Lynch, 1980; Nelson, 1990; Niemi and Häggman, 2002).

However, the actual effects of fermentation on the agronomic and nutritional qualities of cucurbit seeds seem to vary greatly, depending mainly on species, fruit age and fermentation duration (Nerson and Paris, 1988; Nerson, 1991; 2002, 2004). Nienhuis and Lower (1981) reported that longer fermentation of cucumber seeds can decrease the germination percent and rate. Examining the relationship between fruit age at harvest and fermentation in melon, cucumber and watermelon, Edwards et al. (1986) and Nerson (1991) noted that germination of seeds from fully-ripe fruits was not affected by fermentation. The authors showed that fermentation increased the germination percent of immature seeds from cucumber, melon, and watermelon, whereas this decreased in squash.

It is worth noting that from the studies addressing the influence of fermentation on cucurbit seed germination, those examining the fermentation methods are scant (Nienhuis and Lower, 1981). Fermentation methods are widely reported to influence microbial diversity and activity (Silva et al., 2000; Nyanga et al., 2007).

In previous investigations, using the oleaginous type of the bottle gourd, we identified the fruit maturity stages at which seed germination and the level of percentage, macronutrients, mineral elements, and vitamins are at their highest values. We report herein results obtained from a study aimed at determining the influence of seed fermentation method on germination and seedling vigor in two cultivars of the oilseed *L*. *siceraria*.

MATERIALS AND METHODS

Plants materials and treatments

Open-pollinated accessions from two edible-seeded *L. siceraria* cultivars, recognizable by the fruit shape (blocky or round) were used. Seeds from the round fruit cultivar are characterized by the presence of a cap on the distal side whereas those from the blocky fruit cultivar lack this cap (Figure 1). Both cultivars were obtained from the cucurbit germplasm of the university of Nangui Abrogoua (Abidjan, Côte d'Ivoire) where they are recorded by the alphanumerical codes NI304 and NI195 for the round- and blocky-fruited cultivar, respectively. The experiments were conducted in two separate years. In Experiment 1, the plants were cultivated during the second growing season of 2006 (from September to December) and seed germination and vigor were evaluated in February, 2007. In Experiment 2, the plants were grown in the same month in 2007 and were evaluated in February 2008 at the experimental station of the University Nangui Abrogoua (Abidjan, Côte d'Ivoire). In each experiment, the harvested fruits were stored at the farm for two weeks before fermentation tests started.

For each cultivar, and year, 40 fruits were selected to constitute four samples of 10 fruits, corresponding to four fermentation treatments: 1) Control (unfermented seeds); 2) seeds were fermented by exposing the cut fruits in ambient air in the field (SFA); 3) seeds were fermented by packing the cut fruits in a transparent plastic bag that was exposed in ambient air in the field (SFB); and 4) seeds were fermented by packing the cut fruits in a transparent plastic bag that was buried 30 cm depth (SFD). Fermentation started two weeks after harvest for all the selected fruits. The three fermentation processes are those in general used by rural farmers from Sub-Sahara African and Asia countries (Okoli 1984; Hopkins et al., 1996; Nerson 2002). All the 40 fruits used in this study were harvested 50 days after anthesis considered elsewhere to be the normal time for seed harvest in the two cultivars examined. During the trials, the daily temperature averages in ambient air (SFA), plastic bag containing cut fruits and exposed in the field (SFB), and plastic bag containing cut fruits and buried 30 cm depth (SFD) were 29, 32 and 26°C, respectively.

Seeds from the control samples were directly extracted, washed with tap water, and sundried in ambient air until attaining 6 to 7% moisture. Seeds from the other treatments were extracted after a 10-day fermentation period (practice widely used by farmers) then washed and dried until 6-7% moisture. After drying, seeds with similar weights (310 ± 76 mg) were selected for germination and vigor evaluation. This mean was determined on the basis of the individual weight of 750 seeds per cultivar.

Experimental design for seed germination and vigor evaluation and data collection

Seed germination and vigor were evaluated in an on farm trial using a completely randomized complete block design with two blocks and five replications (plots of 1 m × 0.5 m). Blocks were spaced by 3 m. Each plot received 20 seeds, resulting into 100 seeds per treatment per block. The seed samples were sown at 3 cm depth and a spacing of 7 x 7 cm. Sowings were done on raised beds (30 cm height mounds). The rainfall, relative humidity, and temperature were respectively 107.19 mm, 93%, and 28°C, in Experiment 1 and 192.28 mm, 87%, and 25°C, in Experiment 2.

Seed germinability was evaluated using the seed germination percentage (GeP). Seeds were considered as germinated when the cotyledons appeared above the ground level. The seeds sown were surveyed daily for 14 days (ISTA, 1996). Seed vigor was examined using the following parameters: germination speed index (GSI), seedling emergence percentage (EmP), emergence speed index (ESI), shoot length (SSL, measured with a ruler after digging up the

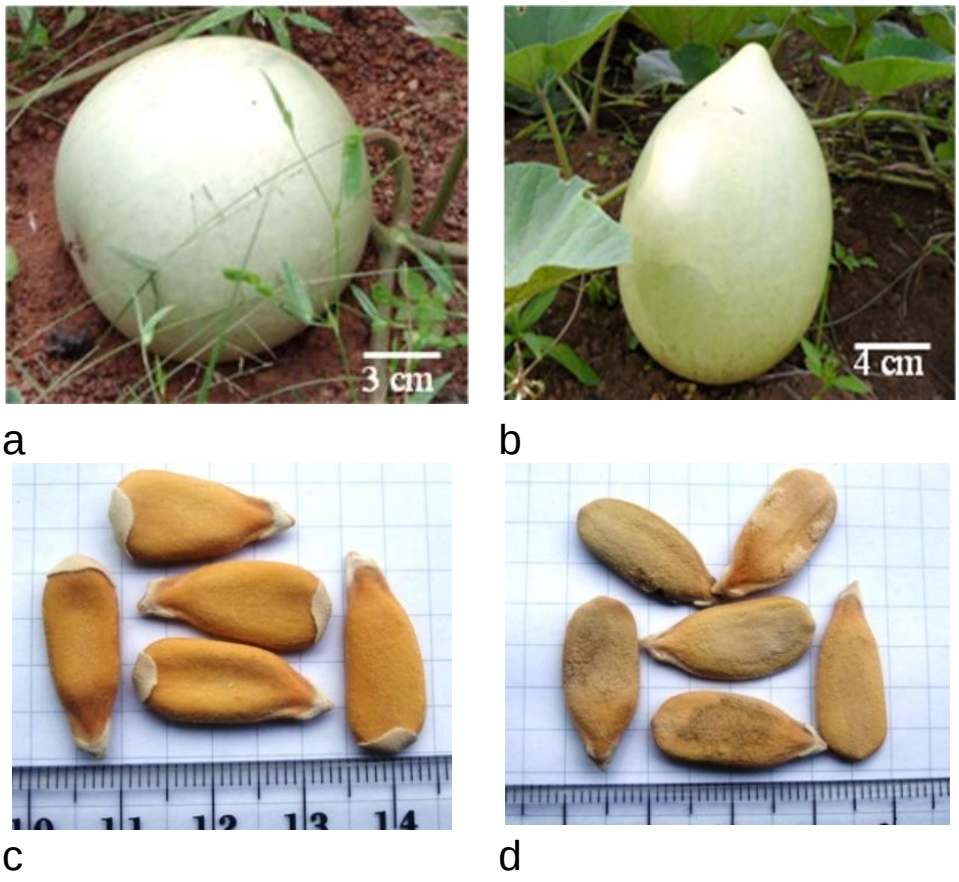

Figure 1. Fruit and seeds of the edible-seeded *Lagenaria siceraria*. a: stem with round fruit; b: stem with blocky fruit; c: seed of the round-fruited cultivar; d: seed of the blocky-fruited cultivar.

emerged seedling), and seedling dry biomass (SDB, measured after drying the seedling to constant weight). A seedling was considered emerged when its two cotyledonary leaves were completely opened (Koffi et al., 2009). The GSI and ESI were calculated on the basis of the procedure used by Maguire (1962) according to the following equation:

$$\text{GSI or ESI} = \frac{X_1}{N_1} + \frac{X_2}{N_2} + \cdots \frac{X_n}{N_n}$$

Where, X_1, X_2, and X_n represent the numbers of germinated seeds or emerged seedlings on the first, second, and last count; N_1, N_2, and N_n corresponding to the numbers of days on the first, second, and last count.

Data statistical analysis

Percentage data were arcsin-transformed before analysis (Little, 1985) but untransformed data were used to calculate means to present the results. Multivariate analysis of variance (MANOVA) appropriate for three-way fixed model was performed using SAS statistical package (SAS, 2004) to check difference between the variable means for each factor tested (fermentation method, cultivar, and time of experiment). For each trait, when the null hypothesis related to the MANOVA was rejected, Duncan's multiple range tests were carried out to identify significant differences among the means of the parameters examined, according to fermentation method, cultivar, experiment, and interactions. All Duncan's multiple range tests were carried out at α = 0.05 significance level.

RESULTS

Global effect of analyzed factors

MANOVA revealed significant influence of the fermentation method, cultivar and experiment. Also, all the three double interactions as well as the triple interaction were significant. Nevertheless, for most of the six traits examined, the trends of results related to single effects of fermentation method and cultivar did not change through the two experiments. Thus, data for these two factors were polled over experiments and only the means are presented (Table 1).

Effect of analyzed factors on seed germination and vigor

The results of each analyzed factor (experiment period,

Table 1. MANOVA results of experiment period, fermentation method, cultivar and their different interaction on seed germination and seedling vigor in the oleaginous *Lagenaria siceraria.*

Factor	DF	*F*	*P*
Experiment	1	67.14	<0.001
Cultivar	1	17.03	<0.001
Method	3	14.73	<0.001
Experiment ×Cultivar	3	3.13	<0.001
Cultivar × Method	7	12.27	<0.001
Experiment × Method	3	11.27	<0.001
Experiment × Cultivar × Method	15	18.43	<0.001

fermentation method and cultivar) and their different interactions on seed germination and seedling vigor are shown in Table 2.

Variation of seed germination and vigor with respect to experiment

For both cultivars, the highest values of the six traits examined were obtained during the Experiment 2 (February, 2008). Thus, high proportions of seeds germinated, gave longest and heaviest shoots in February 2008 experiment, regardless of the fermentation process and cultivars used.

Influence of fermentation process on seed germination and vigor in each cultivar

The parameters expressing seed viability (germination percentage and speed index) and vigor (emergence percentage, emergence speed index, shoot length and dry biomass) were significantly ($P < 0.001$) influenced by fermentation method in each cultivar. The highest values of all the six parameters measured were obtained when seeds were fermented after cutting and packing the fruits into plastic bag, and then hidden under ground in 30 cm depth (SFD). These values were followed in decreasing order by treatments SFB, SFA, and the control.

Response of cultivar to seed germination and vigor

Seed germination and seedling emergence percentages did not vary significantly with cultivar (Table 2). Contrarily to this, the speed of seed germination and seedling emergence, as well as the length and biomass showed highly significant variation according cultivars. The highest speeds of seed germination and seedling emergence were observed in the round-fruited cultivar. Accordingly, significantly high shoot length was obtained with the same cultivar. However, the seedling dry biomass was higher in the blocky-fruited cultivar.

Interaction between cultivar and the fermentation method during each experiment

Although there was a significant effect of fermentation method leading to high values with SFD method in each cultivar, the comparison of both cultivars indicated that GeP, EmP, and SSL did not significantly vary from one cultivar to the other during each experiment. However, GSI, ESI and SDB varied significantly according to cultivars (Table 2). Indeed, even for the best fermentation method (SFD), seedling emerged more rapidly (ESI) in round-fruited cultivar (RFC) while the heaviest seedling (SDB) were obtained with blocky-fruited cultivar (BFC). Even if cultivars showed small difference in germination and vigor, fermentation methods have similar effect on them. Independently of these two factors, the weather during experiment has significant effect on germination and vigor.

DISCUSSION

Techniques used to improve seedling establishment are widely documented (Taylor and Harman, 1990; Copeland and McDonald, 2001). One of the main factors commonly considered to improve seed quality in the continuously flowering fleshy-fruited crops such as cucurbits is seed fermentation (Nerson and Paris, 1988). Seed fermentation efficiency is widely demonstrated but seems to be linked to conditions (that is, method) in which it takes place (Nienhuis and Lower, 1981; Nerson and Paris, 1988; Demir and Samit, 2001; Nerson, 2002; 2007). That is the case in our study.

Seed quality response to fermentation method has been examined in the oleaginous bottle gourd with respect to cultivar and month of experiment. The results show that individual and combined effect of these three factors (fermentation method, cultivar and experiment period) influenced seed germination and vigor. The analysis of these results showed that whatever was extracted in the procedure, seeds obtained from each method germinated and produced at least vigorous seedlings in both cultivars. This proved that seeds used during this study were mature, but compared to control

Table 2. Interaction effect of time of experiment, cultivar and seed fermentation method on the seed germination and seedling vigor in oleaginous *Lagenaria siceraria*.

Experiment	Cultivar	Fermentation method[1]	Parameters[2]					
			GSI[3]	ESI	GeP (%)	EmP (%)	SSL (mm)	SDB (mg)
Experiment 1 (February 2007)	Round-fruited	SFD	3.64 ± 0.05^{d}	2.12 ± 0.05^{bcd}	81.00 ±2.56^{bcde}	79.50 ± 2.63^{bcd}	89.66 ± 1.10^{b}	5.48 ±0.11^{d}
		SFB	3.08 ± 0.04^{e}	1.93 ± 0.05^{d}	74.50 ±2.41^{defg}	72.50 ± 2.01^{def}	87.05 ±0.99^{cd}	4.76 ± 0.11^{f}
		SFA	2.78 ± 0.07^{efg}	1.52 ± 0.08^{f}	67.00 ± 3.35^{g}	65.50± 3.91^{fg}	85.43 ± 1.22^{d}	4.80 ± 0.12^{ef}
		Control	1.95 ± 0.04^{h}	1.35 ± 0.10^{f}	54.50 ± 1.74^{h}	51.00 ± 2.08^{hi}	81.68 ± 1.34^{ef}	4.33 ± 0.14^{g}
	Blocky-fruited	SFD	3.74 ± 0.14^{c}	1.94 ± 0.09^{d}	86.50 ± 2.48^{abc}	84.50 ± 2.52^{abc}	73.22 ± 0.68^{h}	6.76 ± 0.10^{b}
		SFB	3.15 ± 0.12^{d}	1.57 ± 0.09^{f}	79.00 ± 4.52^{cdef}	77.00 ± 4.29^{cde}	64.83 ± 0.86^{i}	5.16 ± 0.09^{de}
		SFA	2.16 ± 0.18^{h}	1.50 ± 0.09^{f}	71.00 ± 4.82^{fg}	69.00 ± 4.64^{efg}	60.38 ± 0.84^{j}	4.69 ±0.10^{f}
		Control	1.97 ± 0.12^{h}	1.06 ± 0.12^{g}	49.00 ± 5.04^{h}	44.00 ± 4.33^{i}	57.67 ± 1.00^{j}	4.58 ± 0.12^{fg}
Experiment 2 (February 2008)	Round-fruited	SFD	4.71 ± 0.11^{a}	2.44 ± 0.07^{a}	95.00 ± 3.16^{a}	92.50 ± 2.81^{a}	84.62 ± 1.05^{de}	7.59 ± 0.20^{a}
		SFB	4.27 ± 0.18^{ab}	2.26 ± 0.09^{abc}	90.83 ± 3.00^{ab}	88.33 ± 3.57^{ab}	76.35 ± 1.08^{g}	5.79±0.12^{c}
		SFA	4.63 ± 0.15^{a}	2.43 ± 0.07^{a}	85.83 ± 1.54^{abc}	83.33 ± 1.67^{abc}	72.38 ± 1.17^{h}	5.45 ± 0.20^{de}
		Control	2.92 ± 0.15^{efg}	1.86 ± 0.11^{de}	68.33 ± 3.80^{g}	59.17± 3.52^{gh}	66.85 ± 1.09^{i}	4.60 ± 0.20^{fg}
	Blocky-fruited	SFD	4.30 ± 0.19^{ab}	1.99 ± 0.12^{cd}	93.33 ± 3.07^{a}	90.83 ± 2.39^{a}	93.04 ± 0.79^{a}	6.88 ± 0.14^{b}
		SFB	4.12 ± 0.22^{bc}	2.44 ± 0.09^{a}	86.67± 3.33^{abc}	85.00 ± 4.08^{abc}	89.10 ± 0.92^{bc}	6.33 ± 0.17^{b}
		SFA	3.72 ± 0.24^{cd}	2.31 ± 0.12^{ab}	85.00 ±1.83^{abcd}	82.50 ±2.14^{abcd}	85.36 ± 0.83^{d}	5.46 ± 0.06^{de}
		Control	2.60 ± 0.18^{g}	1.57 ± 0.08^{ef}	72.50 ± 5.59^{efg}	66.67± 6.15^{efg}	80.72 ± 0.97^{f}	5.07 ± 0.11^{e}
Statistic test results	*F*		5.49	3.51	4.65	4.01	4.48	2.49
	P		<0.001	0.003	<0.001	<0.001	<0.001	0.017

[1] Control: unfermented seeds; SFA: seeds fermented by exposing the cut fruits in ambient air in the field; SFB: seeds fermented by packing the cut fruits into plastic bag that was exposed in ambient air in the field; SFD: seeds fermented by packing the cut fruits into a plastic bag that were buried at 30 cm depth.
[2] GSI: germination speed index; ESI: emergence speed index; GeP: germination percentage; EmP: emergence percentage; SSL: seedling shoot length; SDB: Seedlings dry biomass.
[3] In each column, values with the same superscript letter are not significantly different from each other ($P < 0.05$) using Duncan's multiple range test.
NB: Rainfall in the Experiment 1 was low (107.19 mm) compared to this obtained in the Experiment 2 (192.28 mm).

method (unfermented seeds), all the fermented seeds (SFA, SFB and SFD) showed better germination and vigor. It means that, apart from facilitating extraction of seeds firmly encrusted in the fruit pulp, fermentation improves their germination and vigor (Edwards et al., 1986; Nerson and Paris, 1988; Taylor and Harman 1990). This can partially explain why peasants always ferment cucurbits seeds before extracting them (Nerson, 2007). Comparison of our fermentation methods to each other showed that seeds obtained from closed media (that is, SFB and SFD methods) exhibit better germination and vigor than those fermented at ambient air (SFA).

This difference of seed germinability and vigor observed in *L. siceraria* both cultivars tended to prove that although fermentation improves seed quality, its efficiency depends on condition in which it takes (Nienhuis and Lower, 1981; Nerson and Paris, 1988; Demir and Samit, 2001; Nerson, 2002, 2007). In addition, SFA method being the easiest to apply and widely used by peasant (Okoli 1984; Hopkins et al., 1996; Nerson, 2002) we can assumed that our fermentation methods (SFB and SFD) are more useful than the peasants one (SFA) in seeds quality improvement of this species. Moreover, comparison of our closed media methods (SFB and SFD) revealed that best germination and vigor were obtained with seed fermented in anaerobic, darkness, and relatively low temperature (26°C) conditions (that is, SFD). High performance of the SFD treatment in *L. siceraria* seed quality improving might be due to

single or combined effects of darkness and the relatively low temperature (Nienhuis and Lower, 1981; Demir and Samit, 2001; Woo and Song, 2010). Several studies proved that, for each species, speed and result of the fermentation process largely depend on the temperature and its application duration (Demir and Samit, 2001). For our oleaginous *L. siceraria*, good quality of seed was obtained after incubating at 26°C in dark as reported (24-27°C) in tomatoes (Nienhuis and Lower, 1981; Nerson and Paris, 1988; Nerson, 1991; 2002). Darkness could have favored the metabolic process and/or the development of microorganisms involved in seed fermentation (Madigan et al., 1980; Teramoto et al., 1993). Anaerobic medium, water saturation, and reduced air conditions seemed to favor the proliferation and the activity of microorganisms involved in fermentation (Nienhuis and Lower, 1981; Nerson and Paris, 1988; Silva et al., 2008; Stringini et al., 2009). Beneficial microorganisms developed on seeds during the fermentation process might promote seedling establishment or provide seed borne diseases control (Beaulieu et al., 2004; Bennett and Whipps, 2008). Indeed not all of the various microorganisms proliferating during cucurbit crops seed fermentation (Leben, 1981; Bankole, 1993) have deleterious effects on their germinability. Another hypothesis of the beneficial effect of fermentation on *L. siceraria* mature seeds germinability could also be attributed to cucurbitacin (germination inhibitor) lifting during the process (Nerson and Paris, 1988; Nerson, 1991; 2002; Martin and Blackburn, 2003).

In our study, seed germination and seedling emergence percentages did not vary significantly between both cultivars following seed fermentation method. Even if studies examining differences between crop plant cultivars for seed germination and vigor following fermentation procedure are scant, investigations on the variation of seed germination among cultivars in a plant species are extensive in literature (Silvertown, 1984; Ellison, 2001; Cisse and Ejeta, 2003). Difference between both cultivars of *L. siceraria* could be due to a genetic control of seed germination in several crop plants (Ecker et al., 1994; Sadeghian and Khodaii, 1998; Dias et al., 2011).

Contrarily to germination and emergence percentages, significant variations were noted between cultivars for the speed of seed germination and seedling emergence, as well as the seedling length and biomass; the best performances being observed in the round-fruited cultivar (RFC). This difference between both cultivars for the seedling vigor could be attributed to the difference in their agronomical performances, rather than fermentation method or seed reserves. In fact, the seeds used in this study were of similar weights (310 ± 76 mg), suggesting their similar contents in reserves contrarily to several plant species for which a large amount of seed reserves is used to form a certain size of stem and the necessary number of leaves to set the plant up for photosynthesis during germination and early stages of seedling growth (Wanasundara et al., 1999; Ichie et al., 2001; Kolb and Joly, 2010). Furthermore, we noted from seed regeneration trials that compared to the blocky fruit cultivar (BFC), the RFC usually shows more vigorous vegetative growth and flowers about two weeks earlier.

Independently of the fermentation process and cultivar used, the best seed and seedling qualities were observed in Experiment 2 (February 2008). Such differences could be explained by the difference in rainfall amounts between both experiment periods. Indeed, the rainfall amount in February 2008 (192.28 mm) was about twice that of February 2007 (107.19 mm). It has been proven for several plant species that seed reserves are easily mobilized and seedlings grow more quickly when soil is wet (Bouaziz and Hicks, 1990; Evans and Etherington 1990; Bochet et al., 2007). Although the bottle gourd is well adapted to water deficit, appropriately warm and wet soils are necessary during its germination and early stages of growth (Zoro Bi et al., 2003; Olson et al., 2009). It thus appeared that regardless of cultivar, wet soil is necessary for an on farm reliable evaluation of seed fermentation method in the bottle gourd *L. siceraria*.

The interaction effect of cultivar and seed fermentation method was significant for three vigor parameters (GSI, ESI, and SDB). However, the trends in seed germinability with respect to cultivars and fermentation methods were not quite contrasted, the best values of the parameters analyzed being obtained when seeds were fermented after cutting and packing the fruits into plastic bag, and then hidden under ground at 30 cm depth (SFD). A similar trend could be noted from the examination of the triple interaction effects on seed germination and vigor. Indeed, except for the seedling shoot length (SSL), the highest values of parameters analyzed were obtained in Experiment 2, regardless of the cultivars and treatments. The fermentation method thus appeared as the main factor to assess in order to produce best quality seed in the oleaginous bottle gourd. However, since high rainfall amount enhanced seed germination and vigor, wet soil is also necessary for an on farm reliable evaluation of seed or for crop establishment in the bottle gourd *L. siceraria*.

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Full Length Research Paper

Direct shoot regeneration via organogenesis in chieh-qua (*Benincasa hispida* Cogn. var. Chieh-qua How)

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A plant regeneration system was established from cotyledon explants of chieh-qua (*Benincasa hispida* Cogn. var. Chieh-qua How). To obtain optimal conditions of adventitious shoot induction, the cotyledon explants were excised from seedlings of different genotypes as well as seed germination conditions, and then cultured on media containing different concentrations of 6-benzylaminopurine (6-BA)/1-naphthaleneactic acid (NAA). Among the eight genotypes, the highest rate of shoot regeneration was obtained from the cotyledons of inbred line A39. The adaxial portion of cotyledons of seedlings cultured for 3 days in darkness and 1 day in light was the appropriate explants for adventitious shoot organogenesis. The highest frequency of adventitious shoot organogenesis (52.2%) and mean number of shoots per explant (4.2) were achieved on Murashige and Skoog (MS) medium supplemented with 6 mg l^{-1} 6-BA and 0.2 mg l^{-1} NAA. Adventitious shoots were observed to regenerate directly from cotyledons rather than from calli. A medium supplemented with $AgNO_3$ was not beneficial for shoot induction. Adventitious shoots were elongated in MS medium supplemented with 3 mg l^{-1} 6-BA and 0.2 mg l^{-1} NAA. Elongated shoots were rooted in ½ MS medium with 0.5 mg l^{-1} indole-3-acetic acid (IAA). Regenerated plantlets were transferred to a greenhouse for about 1 month, and subsequently transplanted into an open field.

Key words: *Benincasa hispida*, chieh-qua, adventitious shoot, genotypes, plant regeneration.

INTRODUCTION

Chieh-qua (*Benincasa hispida* Cogn. var. Chieh-qua How), also known as hairy melon, fuzzy gourd, hairy gourd and moa qua (Cantwell et al., 1996), is a member of the Cucurbitaceae family. This family consists of many plants cultivated for their edible fruits. Other well-known members of this gourd family include cucumber, watermelon and squash.

Chieh-qua is an Asiatic crop widely farmed throughout southern China and Southeast Asia with a cultivation history of more than 300 years. There is approximately 15 000 ha of land annually cultivated for Chieh-qua in the Guangdong province of China alone.

In recent years, diseases such as *Fusarium* wilt and *Phytophthora* blight have greatly reduced the cultivation yield of chieh-qua. Breeding for disease resistance has been one of the primary objectives of chieh-qua improvement. In addition to conventional breeding methods, plant biotechnology techniques are expected to contribute to the improvement of disease resistance by means of *in vitro* selection and genetic transformation. An efficient plant regeneration system is a prerequisite for using somaclonal variation techniques and gene transfer technology.

Plant regeneration are affected by factors such as plant

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genotype, explant type, seedling age, culture medium, concentration and a combination of plant growth regulators (PGRs). Up to now, some efficient plant regeneration protocol has been established in some crops of Cucurbitaceae (Chaturvedi and Bhatnagar, 2001; Soniya and Das 2002; Kintzios et al., 2002; Curuk et al., 2002; Lee et al., 2003; Ananthakrishnan et al., 2003; Sultana et al., 2004; Akasaka-Kennedy et al., 2004; Thomas and Sreejesh, 2004; Kathiravan et al., 2006; Selvaraj et al., 2007; Vasudevan et al., 2007a; Suratman et al., 2010). However, the regeneration of Cucurbitaceae crops is not accomplished like other model plants. Our preliminary study provided a regeneration protocol of cotyledon explants of chieh-qua (He et al., 2007). Nevertheless, the frequency of shoot organogenesis was too low (26.6%) to meet the requirement of bio-technique operation.

Accordingly, the objective of the present study was to establish an efficient and stable regeneration system via shoot organogenesis from cotyledon explants of chieh-qua. Special attention was given to the composition of culture medium, genotypic and seedling stage. We also focused on clarifying the effectiveness of $AgNO_3$ in shoot induction from cotyledon explants in chieh-qua.

MATERIALS AND METHODS

The seeds of chieh-qua were provided by the Vegetable Research Institute, Guangdong Academy of Agricultural Sciences. The mature seeds of eight chieh-qua inbred lines, namely, A39, A19, A10, A02, A14, A12, A06 and B05, were used.

Seed sterilization and explants isolation

After the removal of seed coats, the seeds were surface sterilized in 75% (v/v) ethyl alcohol for 30 s followed by 0.1% mercuric chloride ($HgCl_2$) from 7 to 8 min, and then rinsed five times with sterile distilled water. The sterilized seeds were placed in Petri dishes with filter paper and water for germination (Figure 1A). The dishes were autoclaved at 121°C for 20 min before use. The seeds were germinated in darkness for 2, 3, 4 or 5 days at 28 ± 2°C, respectively and then transferred to light (30 µmol m^{-2} s^{-1}) for 1 day (Table 3). Cotyledons from seedlings of different ages were excised as explants.

Media for adventitious shoot induction

Adaxial portions of cotyledons were implanted in Petri dishes with Murashige and Skoog (MS) medium containing different concentrations and combinations of 1-naphthaleneactic acid (NAA; 0, 0.2, 0.4 and 0.6 mg l^{-1}) and 6-benzylaminopurine (6-BA; 4, 6 and 8 mg l^{-1}). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. 0.7% agar and 3.0% sucrose was added to each medium. Cultures were maintained at 25 ± 1°C and 12 h photoperiod with a light intensity of 30 µmol $m^{-2}s^{-1}$. The effect of various concentrations of $AgNO_3$ (0, 0.05, 0.1, 0.5 and 1 mg l^{-1}) in media on the induction of adventitious shoot containing 6 mg l^{-1} 6-BA and 0.2 mg l^{-1} NAA was investigated. $AgNO_3$ was sterilized by filtration and added to the medium after autoclaving. After 4 weeks, the frequencies of callus induction and shoot organogenesis were recorded.

Media for adventitious shoot elongation and rooting

After 4 weeks of growth in shoot induction media, the adventitious shoots were cultured in an elongation medium containing 3 mg l^{-1} 6-BA and 0.2 mg l^{-1} NAA.

Shoots longer than 2 cm were cut and transferred to ½ MS medium or ½ MS medium with 0.5 mg l^{-1} indole-3-acetic acid (IAA), 0.5 mg l^{-1} NAA and 0.5 mg l^{-1} indole-3-butyric acid (IBA) for rooting. The IAA was sterilized by filtration and added to the medium after autoclaving.

Acclimatization

After 3 weeks of growth in rooting medium, plants with well-developed roots were carefully collected and washed with tap water to remove agar clinging to the roots. The rooted plantlets were transferred to plastic cups containing garden soil and commercial compost (1:1), and then placed in a greenhouse. A high humidity was maintained in the greenhouse until new leaves emerged. After about 1 month, the regenerated plants were planted in the field.

Statistical analysis

A completely randomized design was used in all the experiments. Each treatment consisted of 30 explants (six explants per dish), and each experiment was repeated thrice. Data analyses of the variance and mean values were carried out using Duncan's multiple range tests. Significant differences were determined at the 5% level (Gomez and Gomez, 1984).

RESULTS

Effect of plant growth regulators (PGRs) on shoot induction

The cotyledon explants were cultured on MS medium with different concentrations and combinations of NAA (0 mg l^{-1} to 0.6 mg l^{-1}) and 6-BA (6 to 8 mg l^{-1}) (Table 1 and Figure 1C). The explants were considerably enlarged during the first 3 or 4 days of culture. White friable and non-organogenesis callus occurred at the cut surface of the proximal half of explants after 7 days of culture initiation in all samples, except the explants cultured on the medium without NAA. After 15 days of culturing, small protuberances were observed on the most proximal part of the cotyledon. After about 3 to 4 weeks, adventitious shoots were observed on the cuts of the proximal half of the explants cultured in combinations of 6-BA (4 to 8 mg l^{-1}) and NAA (0.2 to 0.4 mg l^{-1}). The highest frequency of adventitious shoot regeneration (52.2%) was achieved on the medium containing 6-BA (6 mg l^{-1}) and NAA (0.2 mg l^{-1}), but the frequency sharply decreased to 33.3% when the NAA concentration was increased to 0.4 mg l^{-1} (Table 1 and Figure 1D). The shoot regeneration frequencies on media containing 6-BA (4 and 8 mg l^{-1}) and NAA (0.2 and 0.4 mg l^{-1}) had no significant difference as compared to the medium containing 6-BA (6 mg l^{-1}) and NAA (0.6 mg l^{-1}). A shoot regeneration frequency of 3.3% was obtained from the medium containing 6-BA (4 and 8 mg l^{-1}) and NAA (0.6 mg l^{-1}). However, no adventitious shoot was

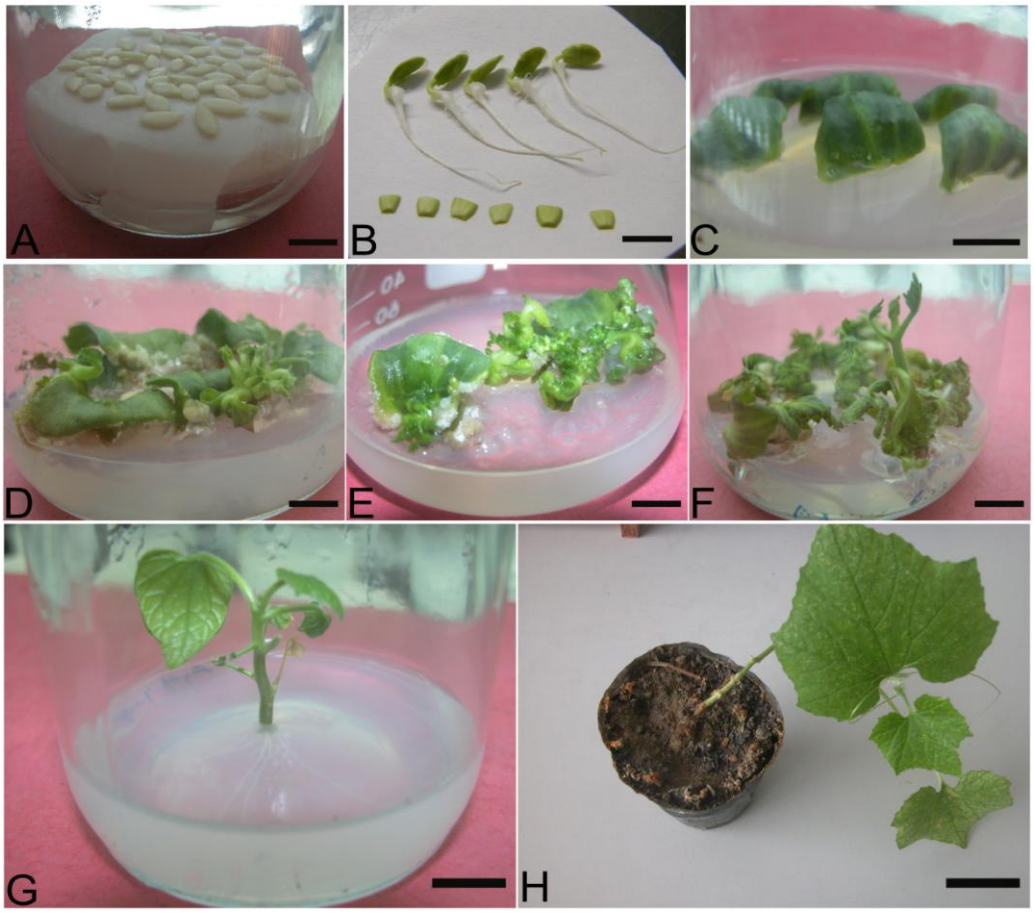

Figure 1. Shoot induction and plant regeneration in chieh-qua. (A) Seeds were germinated on a dish medium with filter paper and water, bar: 1 cm; (B) The adaxial portion of cotyledons of seedlings cultured for 3 days in darkness and 1 day in light was appropriate explants for adventitious shoot regeneration, bar: 1 cm; (C) Cotyledons were cultured on MS medium with 6 mg l^{-1} 6-BA and 0.2 mg l^{-1} NAA, bar: 1 cm; (D) Adventitious shoot regeneration from the proximal portion of cotyledons cultured for 3 weeks on MS medium with 6 mg l^{-1} 6-BA and 0.2 mg l^{-1} NAA, bar: 1 cm; (E) Shoot proliferation occurred when adventitious shoots were subcultured in MS medium with 6 mg l^{-1} 6-BA and 0.2 mg l^{-1} NAA, bar: 1 cm; (F) Adventitious shoots were elongated in MS medium supplemented with 3 mg l^{-1} BA and 0.2 mg l^{-1} NAA, bar: 2 cm; (G) Elongated shoots were rooted in ½ MS medium with 0.5 mg l^{-1} IAA, bar: 3 cm; (H) Rooted plants were acclimatized in a greenhouse, bar: 3 cm.

found on the medium containing 6-BA alone or the combination of 6-BA (4, 6 and 8 mg l^{-1}) and IAA (0.1, 0.3 and 0.5 mg l^{-1}) (data not shown).

Effect of genotype on shoot organogenesis

The cotyledon explants of eight genotypes were tested on the medium with 6 mg l^{-1} 6-BA and 0.2 mg l^{-1} NAA (Table 2). There was no significant difference among the callus formations of all eight genotypes tested; however, the adventitious shoot regeneration frequency and number of shoot per explants varied. The shoot regeneration frequency ranged from 0 to 52.2%. The cotyledon explants of inbred line A39 exhibited the highest rate of shoot differentiation (52.2%) and number of shoots (4.2) on the medium with 6 mg l^{-1} 6-BA and 0.2 mg l^{-1} NAA. The shoot regeneration frequency of inbred lines A02 and A19 had no significant difference. Adventitious shoots were not found in inbred lines A12, A14 and B05.

Effect of seedling age and seed germinating condition on shoot induction

The effect of seedling age and seed germinating condition on adventitious shoot induction was examined. Callus formation was observed in all cultures. However, the shoot regeneration frequencies and number of shoots per explant were influenced by the age of the seedling that provided the cotyledon explants. The cotyledons of 4-day-old seedlings produced a higher frequency of adventitious

Table 1. Effect of plant growth regulators on shoot induction from cotyledon explants of chieh-qua inbred line.

PGRs (mg l^{-1})		Callus formation (%)	Shoot organogenesis (%)
6-BA	NAA		
4	0	0	0^{e}
6	0	0	0^{e}
8	0	0	0^{e}
4	0.2	100	22.2 ± 3.9^{c}
4	0.4	100	25.6 ± 1.9^{c}
4	0.6	100	3.3 ± 0.0^{d}
6	0.2	100	52.2 ± 5.1^{a}
6	0.4	100	33.3 ± 3.3^{b}
6	0.6	100	21.1 ± 5.1^{c}
8	0.2	100	21.1 ± 5.1^{c}
8	0.4	100	20.0 ± 3.3^{c}
8	0.6	100	3.3 ± 0.0^{d}

Proximal cotyledon explants from 4-day-old seedlings of inbred line A39 cultured on MS medium were evaluated after 4 weeks of culture. Means followed by different letters are significantly different at the 5% level. PGRs, Plant growth regulators; 6-BA, 6 benzyl amino purine; NAA, naphthalene acetic acid.

Table 2. Effect of genotype on shoot organogenesis from cotyledon explants of chieh-qua.

Genotype	Callus formation (%)	Shoot organogenesis (%)	No. of shoots per explant
A39	100	52.2 ± 5.1^{a}	4.2 ± 0.2^{a}
A06	100	15.6 ± 3.9^{b}	3.7 ± 0.3^{b}
A10	100	7.8 ± 1.9^{c}	2.3 ± 0.3^{cd}
A02	100	4.4 ± 1.9^{cd}	2.5 ± 0.5^{c}
A19	100	3.3 ± 0.0^{de}	2.0 ± 0.0^{d}
A12	100	0^{e}	0^{e}
A14	100	0^{e}	0^{e}
B05	100	0^{e}	0^{e}

Proximal cotyledon explants from 4-day-old seedlings cultured on MS medium with 6 mg l^{-1} 6-BA and 0.2 mg l^{-1} NAA was evaluated after 4 weeks of culture. Means followed by different letters are significantly different at the 5% level.

shoot regeneration than those of 6-day-old seedlings (Table 3). The seed germinating condition also influenced shoot induction. The highest percentage of shoot regeneration (52.2%) was obtained from 4-day-old seedlings that had been incubated for 3 days in darkness and 1 day in light. The highest number of shoots per explant (5.2) obtained from 6-day-old seedlings incubated for 5 days in darkness and 1 day in light was not significantly different from that of 4-day-old seedlings incubated for 3 days in darkness and 1 day in light (Figure 1B).

Effect of $AgNO_3$ on shoot organogenesis

The medium supplemented with $AgNO_3$ (0.05 to 1 mg l^{-1}) was used to assess the effect of Ag^{+} on shoot organogenesis (Table 4). Cotyledons cultured on shoot regeneration medium became swollen, and adventitious shoots emerged from the cuts of the proximal half of the explants. However, the shoots appeared abnormal and unhealthy. Hyperhydricity was also observed. The $AgNO_3$ concentration highly influenced the frequency of shoot regeneration. The frequencies of callus formation and shoot induction significantly decreased on medium supplemented with $AgNO_3$, but the number of shoots formed per explant had no significant difference from the control.

Shoot proliferation

Shoot proliferation occurred when cotyledon explants with emerging buds were subjected to two successive transfers at an interval of 15-days each in the MS medium with 6 mg l^{-1} 6-BA and 0.2 mg l^{-1} NAA (Figure 1E).

Elongation

The proliferated multiple shoots were separated into small

Table 3. Effect of cotyledon age and seed germination condition on shoot organogenesis from cotyledon explants of chieh-qua inbred line.

Germination time and condition	Callus formation (%)	Shoot organogenesis (%)	No. of shoots per explant
Darkness, 3 days	100	20.0 ± 5.8d	4.0 ± 0.9^{ab}
Darkness, 2 days; light, 1 day	100	38.9 ± 1.9b	4.3 ± 0.1^{ab}
Darkness, 4 days	100	37.8 ± 5.1b	3.7 ± 0.2^{b}
Darkness, 3 days; light, 1 day	100	52.2 ± 5.1a	4.2 ± 0.2^{ab}
Darkness, 5 days	100	14.4 ± 1.9de	3.7 ± 0.7^{b}
Darkness, 4 days; light, 1 day	100	29.7 ± 3.4c	3.3 ± 0.3^{b}
Darkness 6 days	100	13.3 ± 3.0de	3.6 ± 0.6^{b}
Darkness, 5 days; light, 1 day	100	7.8 ± 1.9e	5.2 ± 1.0^{a}

Proximal cotyledon explants from 4-day-old seedlings of inbred line A39 cultured on MS medium with 6 mg l^{-1} 6-BA and 0.2 mg l^{-1} NAA were evaluated after 4 weeks of culture. Means followed by different letters are significantly different at the 5% level.

Table 4. Effect of $AgNO_3$ concentrations on shoot organogenesis from cotyledon explants of chieh-qua inbred line.

$AgNO_3$ (mg l^{-1})	Callus formation (%)	Shoot organogenesis (%)	Number of shoots per explant
0.0	100	52.2 ± 5.1^{a}	4.2 ± 0.2^{a}
0.05	100	13.5 ± 2.1^{d}	3.2 ± 0.2^{ab}
0.1	100	15.5 ± 0.7^{d}	2.8 ± 0.2^{b}
0.5	60	42.0 ± 2.8^{b}	4.1 ± 0.7^{ab}
1.0	16	22.0 ± 2.8^{c}	4.1 ± 0.5^{ab}

Proximal cotyledon explants from 4-day-old seedlings of inbred line A39 cultured on MS medium with 6 mg l^{-1} 6-BA and 0.2 mg l^{-1} NAA were evaluated after 4 weeks of culture. Means followed by different letters are significantly different at the 5% level.

Table 5. Effect of the medium elements on root induction in chieh-qua inbred line A39.

Rooting medium	Rooting response (%)	Callus formation	No. of roots per plantlet
1/2 MS	100	No callus growth	4.3^{b}
1/2 MS + 0.5 mg l^{-1} IAA	100	No callus growth	5.3^{a}
1/2 MS + 0.5 mg l^{-1} NAA	100	Good callus growth	3.0^{c}
1/2 MS + 0.5 mg l^{-1} IBA	100	Slow callus growth	4.7^{ab}

Means followed by different letters are significantly different at the 5% level.

clusters containing two to three shoots. These clusters were transferred to MS medium supplemented with 3 mg l^{-1} 6-BA and NAA 0.2 mg l^{-1}. After culturing for about 4 weeks, the shoots elongated and were available for rooting (Figure 1F).

Shoot rooting

Shoots longer than 2 cm were cut and transferred to ½ MS medium or ½ MS medium with 0.5 mg l^{-1} IAA, 0.5 mg l^{-1} NAA and 0.5 mg l^{-1} IBA for rooting (Table 5). After about 10 days, roots were induced in all four media. The highest number of roots per plant (5.3) was achieved on ½ MS medium containing IAA. Plantlets from ½ MS medium with NAA and IBA had roots with a white callus at the shoot, which reduced their viability. ½ MS medium with 0.5 mg l^{-1} IAA was considered as the appropriate medium for chieh-qua shoot rooting (Figure 1G). The rooted plantlets were transplanted into plastic cups containing garden soil and commercial compost (1:1). The plantlets were maintained in a greenhouse for about a month (Figure 1H), and then planted in a field.

DISCUSSION

An efficient plant regeneration system via organogenesis was established for chieh-qua (*B. hispida* Cogn. var. Chieh-qua How). Cotyledon explants excised from seedlings germinated *in vitro* were used for the regeneration. The present report is probably the first detailed one on the shoot organogenesis of chieh-qua. The explant type, plant genotype, seedling age, culture medium, concentration, and a combination of PGRs were the key factors that influenced shoot organogenesis and subsequent

plant regeneration (Niederwieser and Staden, 1990; Dabauza and Peña, 2001; Ashrafuzzaman et al., 2009; Prakash and Gurumurthi, 2010). Basal MS medium has been found to be the most effective for adventitious shoot organogenesis in Cucurbitaceous cotyledon explants (Kathiravan et al., 2006; Haque et al., 2008; Zhang et al., 2011). Many reports have indicated that 6-BA alone or in combination with auxin is useful for shoot differentiation (Dong and Jia, 1991; Sarowar et al., 2003). In the present study, the combination of 6-BA/NAA was found appropriate for adventitious shoot differentiation from cotyledon explants of chieh-qua. However, the cotyledon explants of chieh-qua did not regenerate adventitious shoots in MS medium with 6-BA alone. Adventitious shoot regeneration in chieh-qua was also found to require cytokinins and auxins. This finding agreed with that reported by Sultana et al. (2004) for watermelon. Selvaraj et al. (2007) have revealed the requirement of a high auxin/low cytokinin ratio for callus formation, and a low auxin/high cytokinin ratio for shoot induction from callus. Thomas and Sreejesh (2004) have reported that the addition of 0.2 µM NAA with 4 µM 6-benzylaminopurine (BAP) was beneficial for adventitious shoot regeneration in the cotyledon-derived callus of ash gourd. When compared with cucumber (Vasudevan et al., 2004; Vasudevan et al., 2007b), watermelon (Sultana and Bari, 2003; Sultana et al., 2004), melon (Curuk et al., 2002; Muruganantham et al., 2002) and squash (Ananthakrishnan et al., 2003; Kathiravan et al., 2006), chieh-qua also needed a high concentration of 6-BA for shoot induction. Such a high concentration may influence shoot proliferation as well as elongation, and could be one of the reasons for the difficulty of regeneration of chieh-qua. Therefore, a balance between cytokinins and auxins is more important for improving the frequency of chieh-qua regeneration.

The cotyledon explants of eight genotypes were tested for adventitious shoot regeneration in the present study. Different regeneration abilities were observed among the genotypes. Hence, adventitious shoot regeneration in chieh-qua was strongly influenced by the genotype. Similar results have been reported in the tissue culture of muskmelon (Molina and Nuez, 1995) and cucumber (Mohiuddin et al., 1997). George et al. (2008) have found that direct or indirect regeneration readily occurred only in some plant species, or may even be restricted to certain varieties within species. This phenomenon may be attributed to the silencing of relevant genes in the heterochromatin. Genotypes with high regeneration abilities should be selected to establish an efficient shoot regeneration system from cotyledon explants in chieh-qua.

In the present study, the shoot regeneration frequency of cotyledons increased after a pretreatment combining darkness with light during seed germination. Similar results have been reported in the cotyledon culture of watermelon (Compton and Gray, 1993). In contrast, Han et al. (2004) have discovered that when compared with light pretreatment, the darkness pretreatment of seedlings decreases the frequency of the adventitious shoot regeneration of cotyledon explants in bottle gourd. Apparently, the effect of alternating darkness and light during seed germination on shoot regeneration from cotyledon varied in different species. Previous studies have shown that the frequency of shoot regeneration was influenced by the seedling age in cucurbits (Compton, 2000; Lee et al., 2003; Krug et al., 2005; Ntui et al., 2009). In the present study, the shoot regeneration frequency of cotyledons from 4-day-old seedlings was higher than those from 5 and 6-day-old seedlings. Dong and Jia (1991) have suggested that young cotyledons are physiologically very active and easily affected by environmental factors, such as exogenous hormones. Therefore, the suitable seed germination condition and seedling age for each species of Cucurbitaceous need to be determined to establish an efficient shoot regeneration system.

$AgNO_3$ inhibits the action of ethylene (Beyer, 1979), which is widely reported to be beneficial for shoot organogenesis of several crops (Burnett et al., 1994; Mohiuddin et al., 1997; Burgos and Alburquerque, 2003; Han et al., 2004; Mohiuddin et al., 2005). In the present study, we observed that the application of $AgNO_3$ negatively affected chieh-qua shoot induction. Callus induction and proliferation were also significantly decreased by high levels of $AgNO_3$. Similar results have been reported in fig leaf gourd (Kim et al., 2010). These results indicate that $AgNO_3$ may have distinct influences on shoot induction. Therefore, we proposed that the use of $AgNO_3$ should be avoided in culture media for chieh-qua shoot induction.

For the shoot induction, we found that the proximal part of the cotyledon showed a higher frequency of adventitious shoot regeneration than the distal part. In fact, no adventitious shoot was found on this part. The proximal part of the cotyledon played a key role in shoot induction. Cells in this part were sensitive to PGRs and had the potential for adventitious shoot formation. Hence, organogenesis was determined by the part of the cotyledon. A similar phenomenon has been observed in some other Cucurbitaceous species (Compton, 2000; Lee et al., 2003; Ananthakrishnan et al., 2003).

According to our results, ½ MS medium with 0.5 mg l^{-1} IAA was appropriate for chieh-qua shoot rooting. Many studies have demonstrated *in vitro* that low-salt, ½ and ¼ MS media promote shoot rooting in cucurbits (Lee et al., 2003; Han et al., 2004; Thomas and Sreejesh, 2004). Auxin is also widely used for root induction in cucurbits, including IAA (Han et al., 2004), NAA (Kathiravan et al., 2006; Kim et al., 2010) and IBA (Sarowar et al., 2003; Krug et al., 2005). All these observations revealed that the shoot rooting response may be affected by the growth medium composition. Differences in rooting response may be attributed to culture conditions or genotypes (Shakti et al., 2007). Therefore, the optimal medium for shoot rooting in an *in vitro* culture of Cucurbitaceous species need to be determined.

In summary, we established a plant regeneration system

via organogenesis from cotyledon explants of chieh-qua, which may contribute to the cell and genetic engineering of this crop. However, some problems were encountered, such as the formation of ill-defined buds and shoot-like structures either resisting elongation or producing rosettes of distorted leaves, which generally do not produce normal shoots. Similar problems have been observed in melon (Gaba et al., 1999) and pepper (Steinitz et al., 1999; Ochoa-Alejo and Ramirez-Malagon, 2001). These problems may be linked to genotypes or the medium for shoot regeneration. Future research will be focused on these areas.

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Full Length Research Paper

Characterization of a new feather-degrading bacterium from *Calotes versicolor* feces

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A total of 842 spore-forming strains were isolated from 221 animal feces samples, in which a new feather-degrading bacterium identified as *Bacillus* sp. 50-3 based on morphological, biochemical and 16S rDNA tests was isolated from *Calotes versicolor* (an agamid lizard) feces. The bacterium can degrade native feather completely at mild conditions and in much shorter time (36 h) when using chicken feather as the sole carbon and nitrogen source. It presented optimum growth and maximum keratinase activity (680±25 U/ml, 36 h) at 37°C and pH 7.0 in feather meal medium. The keratinase of *Bacillus* sp. 50-3 was active on feather keratin as substrate and presented optimum additive quantity of 1.0% (w/v). So the high efficiency of *Bacillus* sp. 50-3 on feather-degradation suggested its potential use in biotechnological processes, especially in decreasing the environment pollution.

Key words: Animal feces, *Bacillus,* chicken feathers, keratinase, optimum conditions.

INTRODUCTION

The micro-organisms and their enzymes play an important role in improving the industrial process. Because the enzyme-catalyzed reactions are highly efficient, easily selective and less pollution, usually they require mild conditions and less energy which lead to the lowering of costs (Cherry and Fidantsef, 2003). And the world market has a big need for industrial enzymes which is estimated to be about 1.6 billion $US, split among food enzymes (29%), feed enzymes (15%) and general technical enzymes (56%) (Outtrup and Jorgensen, 2002). Thus, study on isolating new enzymes and new enzyme-producing strains used in the industrial conversions are significant.

Bacillus species are attractive industrial organisms because of their high growth rates leading to short fermentation cycle times; their capacity to secrete proteins into the extracellular medium and the GRAS (generally regarded as safe) status by the food and drug administration for species, such as *Bacillus subtilis* and *Bacillus licheniformis* (Marcus et al., 2004).

Keratin is the most abundant structural protein in skin, horn, hair, wool and feathers, which is rich in α-helix or β-sheet linked with cystine bridges (Bockle et al., 1995). So the commonly known proteases could not degrade the keratin to a large degree; however, the keratinase purified from different microorganisms and characterized to date all act as proteases have a high level of activity on it. In recent years, there have been many reported microorganisms to produce the enzyme such as *Bacillus* species (Bockle et al., 2005; Kim et al., 2001; Lin et al., 1992; Williams et al., 1990), fungi (Bockle et al., 1995; Chao et al., 2007; Gradisar et al., 2000; Santos et al., 1996) and others (Bernal et al., 2006; Nam et al., 2002; Riessen and Antranikian, 2001; Riffel et al., 2003; Sangali and Brandelli, 2000; Thys et al., 2004). However, there is still a need to find the new strains and enzymes that can be applied in the biotechnological processes involving keratin-containing wastes from poultry and leather industries, especially in the development of non-polluting processes (Onifade et al., 1998; Shih, 1993; Wang et al., 2007).

In this study, we isolated 842 strains of spore-forming

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bacteria from 221 animal feces samples obtained from Beijing Zoo, China and one strain identified as *Bacillus* sp. 50-3 was found to have high keratinase activity. Some features, such as growth conditions, keratinase producing conditions were also determined.

MATERIALS AND METHODS

Chemicals

Chemicals used in the bacterial cell cultivation and taxonomic studies were from Oxoid Ltd (Basingstoke, UK) and Merck AG (Darmstadt, Germany); Azokeratin was synthesized based on the method described elsewhere (Riffel et al., 2003); Chicken feather was from Beijing Huadu chicken factory (Beijing, China).

Sample collection

Samples were collected from Beijing Zoo, China. These fresh samples were from 221 animals such as Snow leopard, Siberian Tiger, Sika Deer, Kangaroo, Racoon, Squirrel Monkey, Takin, Addax, Giant Panda, Lizard, Cougar and Cobra, which were all fed in Beijing Zoo. These samples in sterile 100 ml glass bottles were carried back to laboratory.

Strain isolation

Microorganisms were extracted by suspending 1 g of each feces sample in 9 ml sterile 0.9% (w/v) saline solution using the end-point dilution technique. Firstly, the test tube with feces concentration of 0.1 g/ml was treated for 10 min at 80°C to isolate only spore-forming bacteria. 0.1 ml of each dilution from the proper concentration was spread on agar plates containing the feather meal and the resulting plates were incubated for 72 h at 37°C. The feather-meal agar plates contained: [10 g feather meal, 0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g kH_2PO_4, 15 g agar, per litre; pH 7.0 to 7.2].

The colonies with clear zones formed by hydrolysis of feather keratin were evaluated as keratinase producers. The strains whose zone diameter is 5-fold longer than the colony diameter were selected and used in all further investigations.

Growth determination

The inoculum culture is the Luria-Bertani (LB) [10 g bactotryptone, 5 g yeast extract, 10 g NaCl, per litre; pH 7.2-7.6]; 20 ml of the feather meal medium [10 g feather meal, 0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g kH_2PO_4, per litre; pH 7.0 to 7.2] in 100 ml Erlenmeyer flasks were inoculated from a 10^6 colony forming units (CFU)/ml culture and then cultivated by shaking at 150 rpm for 72 h. Growth of the isolation was tested within the temperature range of 25 to 70°C (25, 37, 45 and 70°C) and pH range of 4.0 to 11.0 (4.0, 5.0, 7.0, 8.0 and 11.0). Bacterial growth was monitored by measuring the CFU/ml, as described elsewhere (Sangali and Brandelli, 2000).

Taxonomic studies

The morphological and physiological characteristics of the isolated bacteria were compared with data from Bergey's manual of determinative bacteriology (Holt et al. 1994).

The strain DNA was extracted from cells after cultivation for 24 using the TIANamp bacteria DNA kit (Beijing TIANGen Biotech, China) according to the manufacturer's instructions. The 16S rDNA gene was amplified by PCR using primers 27f (5´-GAGAGTTTGATCCTGGCTCAG-3´), 530f (5´-GTGCCAGCAGC CGCGG-3´) and 1541r (5´-AAGGAGGTGATCCAGCCGCA-3´), corresponding to *Escherichia coli* 16S rRNA gene position. The PCR products were purified and sequenced by the Beijing HuaDa Gene Study Center, China with the ABI 3730 automated sequencer. The 1,477 bp sequence was submitted to Genbank (accession number EU365432) and was searched for its homologous sequences by BLAST algorithm method. The 16S rDNA sequences were reversed, aligned and compared with similar database sequences using the Clustal X program, version 1.83 (Thompson et al., 1997). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) employing the program molecular evolutionary genetic analysis (MEGA) 3.1 (Kumar et al., 2004). The branching pattern was checked by 1,000 bootstrap replicates.

Enzyme production

The organism was cultivated for 36 h in feather meal medium, from a 10^6 CFU/ml culture. Samples were centrifuged at 10,000×*g* for 10 min and the supernatant fluid used as a crude enzyme preparation. To study cell-associated enzyme, the collected cells were washed twice with 50 mM Tris buffer (pH 8.0) and suspended in the same buffer. The cells were disrupted by sonication for 10 min at 4°C and centrifuged at 10,000×*g* for 10 min. The supernatant fluid was used to determine the enzyme activity.

Enzyme assays

Keratinase activity was assayed with azokeratin as a substrate according to the modified method described by Riffel et al. (2003). The reaction mixture contained 200 µl of enzyme preparation and 1.6 ml of 10 g/l azokeratin in 50 mM glycine/NaOH buffer, pH 10.0. The mixture was incubated for 15 min at 60°C. The reaction was stopped by adding the trichloroacetic acid to a final concentration of 100 g/l. After centrifugation at 10,000×*g* for 10 min, the absorbance of the supernatant fluid was determined at 440 nm. One unit of enzyme activity was the amount of enzyme that caused a change of absorbance of 0.01 at 440 nm for 15 min at 60°C.

Effect of the different substrates on the keratinase activity

Three different substrates were tested in the experiment. The three media contain [10 g of human hair, native chicken feather and the cattle tendon respectively, 0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g kH_2PO_4, per litre; pH 7.0-7.2], 20 ml of each in 100 ml Erlenmeyer flasks was inoculated from a 10^6 CFU/ml culture and cultivated by shaking at 150 rpm for 72 h. The keratinase activity was tested every 12 h.

RESULTS

Isolation of the spore-forming bacteria and selection of the keratin-degrading strain

A total of 221 feces samples from different animals were collected to isolate the spore-forming bacteria. All of 842 pure cultures of spore-forming bacteria were obtained and 196 strains observed the clear zone on feather-meal agar plates. Among them, strain 50-3 was isolated from C*alotes versicolor* (an agamid lizard) feces. The degrading zone diameter of it is about 6-fold longer than the colony diameter. Also, it could degrade the chicken feather absolutely, after 36 h culturing on the native chicken

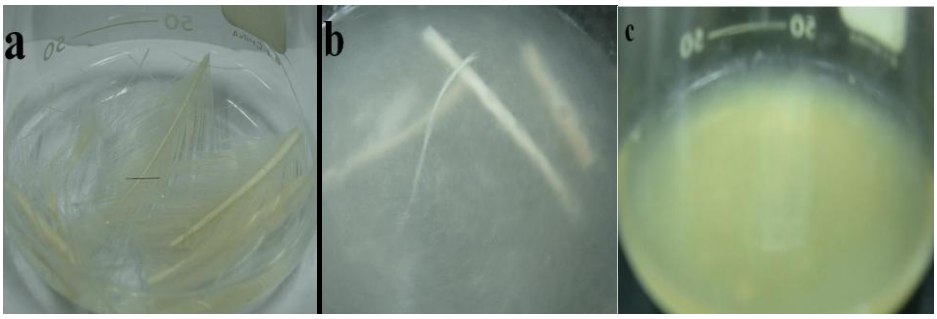

Figure. 1 The native Chicken feather was degraded after 0 h (a), 24 h (b) and 36 h (c), respectively at 37°C by strain 50-3.

feather as the sole carbon, nitrogen and energy source (Figure 1). So the strain 50-3 was chosen for further characterization.

Identification of strain 50-3

The identification of strain 50-3 was based on the morphological, the physiological characteristics and the 16S rDNA tests. Some characters were summarized in Table 1. Together with morphological and physiological characteristics assigned the strain 50-3 to the genus *Bacillus*. The closest species were *Bacillus velezensis* (Cristina, 2005) and *Bacillus vallismortis* (Roberts et al., 1996), which differed from 50-3 in the Tween-80 hydro-lysis and producing arginine dihydrolase, respectively.

The phylogenetic tree based on the comparison of 16S rRNA sequences of reference strains was constructed (Figure 2). The isolate 50-3 formed a highly compact phylogenetic cluster with the following species: *B. velezensis*, *B. vallismortis*, *B. subtilis* and *B. amyloliquefaciens*.

Optimal growth conditions

The strain *Bacillus* sp. 50-3 could grow at 25 to 70°C and pH 4.0 to 11.0, with an optimum at 37°C and pH 7.0. It grew slowly at 25 and 70°C, with less cell number compared with 37 and 50°C. It grew at approximately the same rate at pH 5.0 and 7.0, but more slowly at pH 4.0 and 11.0 (data not shown). Interestingly, the all final pH became around 8.0 after being cultivated for 72 h.

Production of keratinase

During cultivation of *Bacillus* sp. 50-3 in feather meal medium at 37°C, less activity of keratinase was found in the cell lysate and the highest amount of keratinase activity was produced in the supernatant fluid of the culture medium (data not shown). At the optimal growth conditions, the keratinase reached a maximum activity at 36 h, coinciding with the end of exponential phase, then decreased and increased again at 72 h (Figure 3).

The effects of temperature and pH on the production of keratinase were investigated. Maximum enzyme activity was observed during cultivation at 37°C (680±25 U/ml at 36 h), followed by 25°C (465±18 U/ml at 48 h), but less enzyme activity at 50 and 70°C (165 ±36 U/ml at 36 h and 116± 22 U/ml at 24 h, respectively). At 37°C, with the different initial pH, the maximum keratinase activity appeared at different time, especially at pH 4.0, the enzyme activity was higher with increasing cultivation time before 60 h (data not shown) and furthermore, the most proper pH was 7.0. Thus, the maximum keratinase activity was observed at 37°C with initial pH 7.0 and cultivation time 36 h in feather meal medium.

Effect of different substrates on keratinase activity

Three of the different substrates were used to test the effect on keratinase activity (Figure 4). The chicken feather substrate presented greatly increases the keratinase production. On the other hand, the human hair and cattle tendon suggested relatively low enzyme activities.

Effect of different feather meal concentration

The effect of different feather meal concentrations on keratinase activity was investigated. It was seen that the feather meal concentration on the keratinase activity was not important (Figure 5). The optimum concentration was 1.0% (w/v) as the keratinase activity was relatively high.

DISCUSSION

In earlier reports, the spore-forming strains have been isolated from variously ecosystems such as soil, water and food (Lin et al., 1999; Shih et al., 2005). The isolates from animal feces were less reported (Nakada and Ohta, 1999; Swiecicka et al., 2002). In this study, there are so many spore-forming bacteria in animal feces, which is an organic matter-rich environment. The most active strain 50-3 was isolated from *C. versicolor* (an agamid lizard)

Table 1. Morphological and physiological characteristics of the strain 50-3.

Morphological characteristic		Physiological characteristic	
Form	Rods	Catalase	Positive
Size	0.5×1-2 μm	Oxidase	Positive
Gram stain	Positive	Nitrate reduction	Positive
Spore	Positive	Voges-Proskauer test	Positive
Acid from		Citrate	Negative
D-xylose	Positive	Propionate	Positive
Galactose	Negative	alginate	Negative
D-fructose	Positive	Gelatin liquefaction	Positive
Mannose	Positive	Starch hydrolysis	Positive
Mannitol	Positive	Casein hydrolysis	Positive
Cellobiose	Positive	Tween 80 hydrolysis	Positive
Maltose	Positive	Lysine decarboxylase	Positive
Melitose	Negative	Arginine dihydrolase	Positive
D-trehalose	Positive	Urease	Positive
Saccharose	Positive	DNAse	Positive
		Indol production	Positive

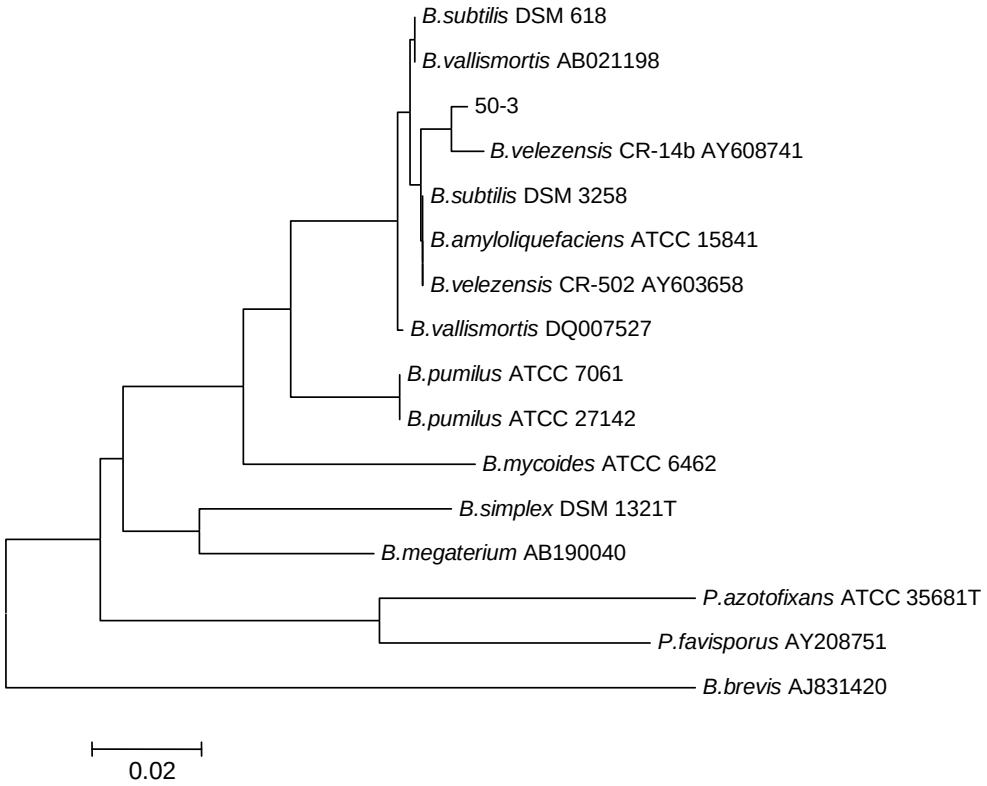

Figure. 2 Strain 50-3 and selected bacteria comparisons of phylogenetic tree based on 16S rDNA sequence. The branching pattern was generated by the neighbor-joining method. The bar indicates the 2% estimated difference in nucleotide sequences.

feces, which could be as a microorganism of environmental origin. As it is known that C*. versicolor* is an agamid lizard found widely in Asia, the main food of which is insects such as crickets, beetles and spiders. Therefore, there could be some microorganisms colonizing its gastrointestinal tract to degrade the insects' scute, which is full of keratin and facilitate efficient digestion (Angelis et al., 2006; Bevilacqua et al., 2003).

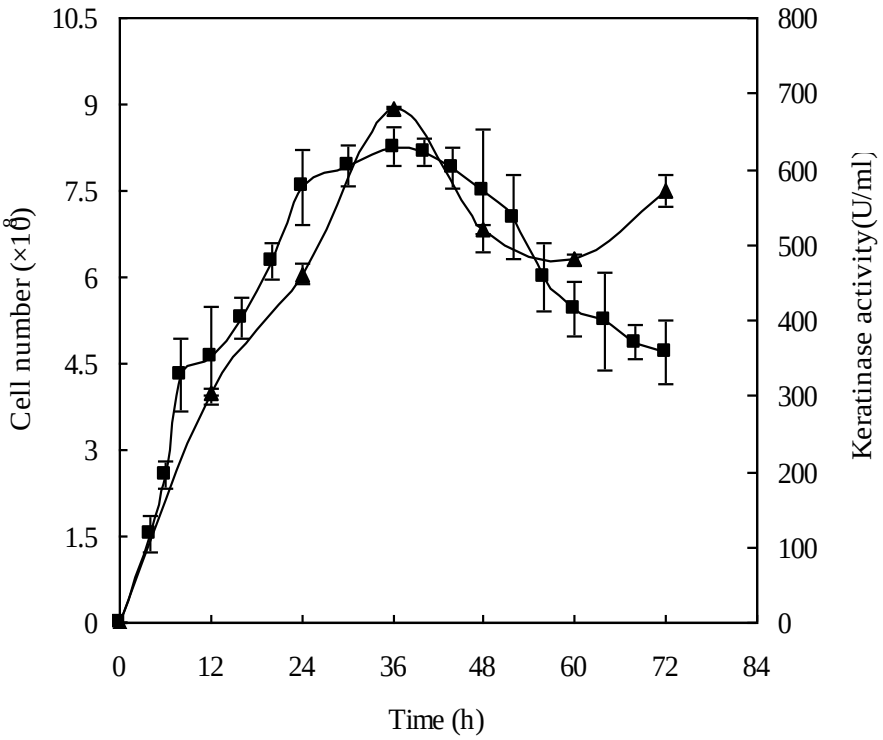

Figure. 3 Time course of the growth and keratinase production of *Bacillus* sp. 50-3 grown on 1.0% feather-meal medium at 37°C by shaking at 150 rpm. Closed diamond: Cell, closed triangle: Keratinase activity. Each point represents the mean±SEM of three independent experiments.

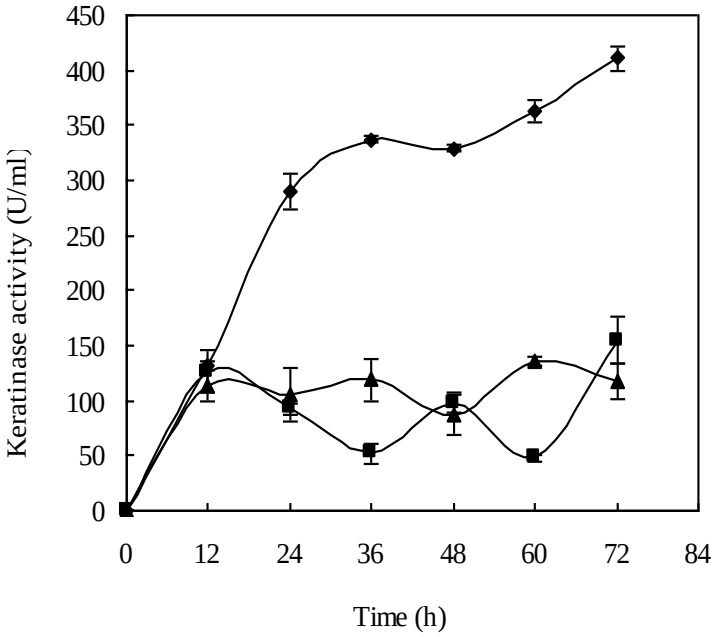

Figure. 4 Effect of three different substrates on keratinase activity from *Bacillus* sp. 50-3. To measure activity, the enzyme was assayed at each 12 for 72 h by the method in above. (closed diamond) native chicken feather; (closed triangle) cattle tendon; (closed square) human hair. Each point represents the mean±SEM of the three independent experiments.

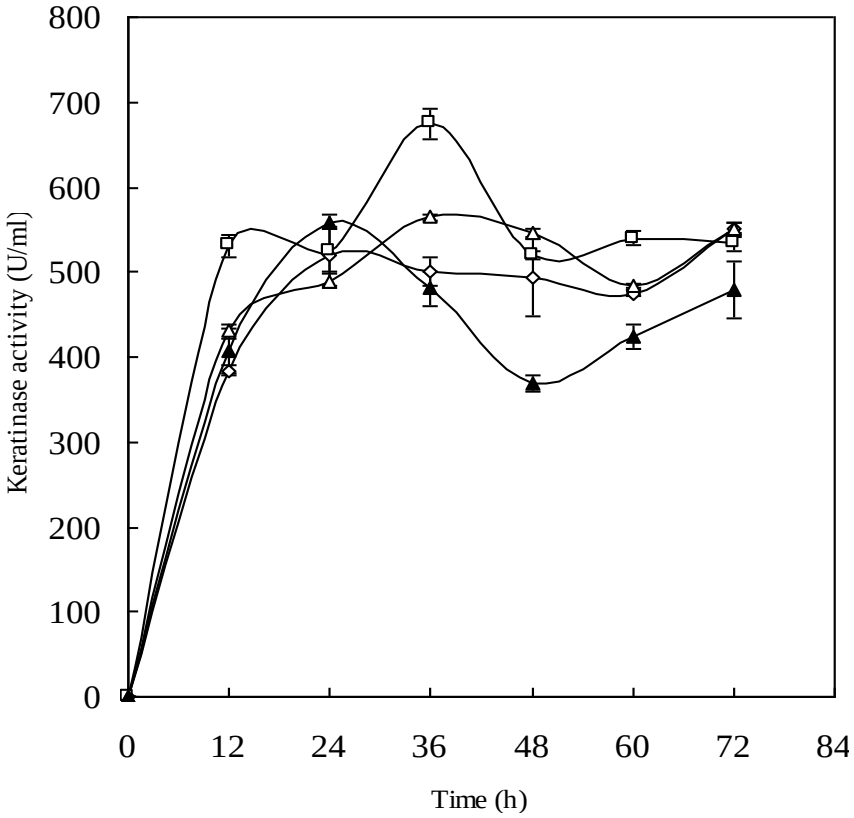

Figure. 5 Effect of different feather concentration on the keratinase activity from *Bacillus* sp. 50-3. To measure activity, the enzyme was assayed at each 12 for 72 h by the method in above. Open diamond 0.5%, open square 1.0%, open triangle 1.5% and closed triangle 2.0%. Each point represents the mean±SEM of three independent experiments.

The morphological and physiological characteristics and the 16S rRNA sequence indicated that strain 50-3 was within the *B. subtilis* group. In a previous paper on taxonomy, species included in the *B. subtilis* group are the following: *B. velezensis*, *Bacillus atrophaeus*, *B. mojavensis*, *Bacillus malacitensis*, *Bacillus axarquiensis*, *Bacillus nematocida*, *Bacillus vallismortis*, *B. subtilis* and *Bacillus amyloliquefaciens* (Roberts et al., 1994, 1996; Wang et al., 2007). The 16S rDNA sequence showed high similarity (>99%) to some strains of *B. velezensis*, *B. vallismortis* and *B. subtilis*, a level of similarity which was greater than the borderline 98.7% for defining bacterial genomic species (Stackebrandt and Ebers, 2006; Stackebrandt and Goebel, 1994). Thus, only the 16S rDNA sequencing was not helpful in determining which species the strain 50-3 belonged to and its accurate identification needed more study. Therefore, the strain was named *Bacillus* sp. 50-3 at present.

Bacillus species are an important source of industrial enzymes which make up about 50% of the total enzyme market (Marcus et al., 2004). But the newly isolated *Bacillus* sp. 50-3 is a novel *Bacillus* strain to degrade feather keratin with high keratinase activity. Compared with most other feather degrading strains (El-Refai et al., 2005; Lin et al., 1999; Nam et al., 2002; Riffel et al., 2003), it could degrade the native feather in much shorter time (36 h) and have a relatively high keratinase activity (680±25 U/ml) at the same conditions.

The strain *Bacillus* sp. 50-3 could grow at wide temperature and pH conditions. During cultivation both at initial low pH 4.0 and high pH 11.0 in feather meal medium, pH would come to around 8.0 which indicated the keratin hydrolysis (Sangali and Brandelli, 2000). At low initial pH, it presented the same result with the previous report (Riffel et al., 2003; Sangali and Brandelli, 2000). And at higher initial pH, the pH would decrease firstly, which might be caused by the keratin degradation and the free carboxyl increases in the medium to balance the pH which was proper for the strain growth.

The keratin is the abundant structural protein in feather and hair (Riessen and Antranikian, 2001) and the keratinase is inducible requiring keratin as an exogenous inducer (El-Refai et al., 2005). Therefore in feather medium, the keratianse could be largely inducible and its enzyme activity is higher than that in hair medium (Figure 4). The hair was difficulty to degrade; this might be caused by the hair keratin has much more α-helix structures which are difficulty to degrade than the feather keratin. Also in cattle

tendon, collagen is the main protein, so the keratinase activity was much lower even the cattle tendon was degraded absolutely. This was different from the result of the *Fervidobacterium islandicum* AW-1 (Nam et al., 2002).

The newly isolated strain 50-3 was a new *Bacillus* strain with high keratinase activity and it was from rather unorthodox sources, which indicated its novel characters. The strain could degrade the native feather in much shorter time at mild conditions. This suggested its potential use in biotechnological processes involving keratin hydrolysis to improve efficiency. In addition, the animal feces are rich in organic matter, which could lead to isolate much more useful microorganisms from them in the environment. Also, the keratianse character is under investigation, as far as the high biotechnological potential of the strain is concerned.

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Characterization of cellulase production by carbon sources in two *Bacillus* species

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The induction of cellulase production in two *Bacillus* spp. was studied by means of measuring cellulase activities under the condition of different carbon sources. The results indicate that cellulase could not be induced by cellulose material as a sole carbon source. Instead, they could be induced by monosaccharide or disaccharide with reducing group. Moreover, the expression of cellulase components was synergistic. When cell wall/envelope enzyme and endoenzyme from two *Bacillus* spp. acted on these inducers, analysis of reaction products by high performance liquid chromatography (HPLC) revealed that cell wall/envelope enzyme and endoenzyme from two *Bacillus* spp. were inactive on these inducers. It also indicated that these inducers entered cells directly and served function of induction.

Key words: *Bacillus*, cellulase, induction, carbon source.

INTRODUCTION

Cellulase is a multi-enzyme complex, regulated and controlled by complicated metabolic process. Low yield and specific activity are major factors in preventing application of cellulase. Therefore, clarifying the induction mechanism of cellulase production and its regulating and controlling principles will be meaningful not only in theory, but also in providing clues and methods to enhance the cellulase yield as well as to design screen model which can select high yield strain. In the past, the studies of inducible and regulating mechanism mainly focused on some microbial strains such as *Trichoderma* sp. The synthesis of cellulase can be induced by many oligomeric and dimeric sugars (Hrmova et al., 1991; Magnelli and Forchiassin, 1999). It was difficult to use a general induction mechanism to explain diversified inductions, though several inducers have been found. For example, Nochure et al. (1993) demonstrated fructose as the best inducer of avicellase in *Clostridium thermocellum*. Bagga et al. (1989) identified lactose as the best inducer of endo-glucanase and cellobiohydolase. Thirumale has been reported

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Figure 1. Morphology of cells of *Bacillus* sp. X18 observed under light microscope (100×).

Figure 2. Morphology of cells of *Bacillus* sp. X10-1-2 observed under light microscope (100×).

Figure 3. Colony and clear halo formed by *Bacillus* sp. X18 on the screening plate stained with Congo red.

Figure 4. Colony and clear halo formed by *Bacillus* sp. X10-1-2 on the screening plate stained with Congo red.

as the best inducer of cellulases in a *Clostridium* sp. (Thirumale et al., 2001). Fructose and cellobiose, among the monomeric and saccharides and disaccharides examined, respectively were found to be the best inducer of cellulase in *Aspergillus niger* (Hanif et al., 2004). Recently, with the rapid development in both biochemistry and molecular biology, along with the intensive application of cellulase in some new fields, many people are interested in the regulating mechanism of cellulase synthesis, being a new focus in the study of cellulase. At the same time, because of the demand of bacteria cellulase, the mechanism of cellulase production will be a research pivot. Some studies showed that cellulases in bacterial and fungal systems are different. Incomparison with the cellulases of the fungi, very little is known about the mechanisms by which bacteria produce cellulose (Wood and Carcía-Campayo, 1990). There is no report on the inducer of *Bacillus* species at present.

The main purpose of this study was to investigate the induction mechanism of cellulase production in two *Bacillus* spp. Preparation and assay of endocellular enzyme and cell wall/envelope enzyme were determined. In addition, the reactions of endocellular enzyme and cell wall/envelope enzyme with inducers were also evaluated.

MATERIALS AND METHODS

Organism and chemicals

Bacillus sp. X18 and *Bacillus* sp. X10-1-2, isolated and screened by the laboratory of Green Chemical Technology of College of Heilongjiang Province, were used in this study. After isolation, the organisms were purified through repeated plating in beef extract-peptone agar media. For the identification of selected isolates, different morphological and cultural characteristics such as size, shape, arrangement, colour, growth on agar plate, agar slants, in liquid or in deep agar media, etc., were observed. The morphology of cells of *Bacillus* sp. X18 and *Bacillus* sp. X10-1-2 are shown in Figures 1 and 2. There were few significant differences among the morphology images of the two strains. As show in Figures 3 and 4, colony and clear halo were formed by *Bacillus* sp. X18 and *Bacillus* sp. X10-1-2 on the screening plate stained with Congo red. Finally, the characteristics were compared with Buchanan and Gibbons (1974) and provisionally identified as *Bacillus licheniformis* and *Bacillus cereus*, respectively. Two bacterial strains were maintained

on beef extract-peptone slants. All chemicals were of analytical grade except acetonitrile which was of chromatographicgrade.

Enzyme production

The medium for enzyme production was prepared by mixing 3.0 g Na_2HPO_4, 2.0 g $(NH_4)2SO_4$, 0.5 g urea, 0.5 g $MgSO_4•7H_2O$, 0.5 g $CaCl_2$, 7.5 mg $FeSO_4•7H_2O$, 2.5 mg $MnSO_4•H_2O$, 2.0 mg $ZnSO_4$ and 3.0 mg $CoCl_2$ in 1 L distilled water, autoclaved for 20 min at 121°C, adding specific carbon source. The concentration of carbon source: cellulose and sugar was 0.5 % (W/V) and 0.1 % (W/V), respectively. The seed culture was developed on beef extract-peptone slants for 48 h at 30°C. Slant culture was washed with 4 ml of sterile water to obtain spore suspension. In order to produce cellulase, 0.1 ml of the spore suspension was inoculated into 150-ml Erlenmeyer flasks containing 30 ml fermentation medium to give a spore concentration of 106 spores per millilitre. The flasks were incubated at 30°C on a gyratory shaker at 170 rpm. Sample flasks in one time replicate were removed for assaying enzyme activities present in the culture every 24 h intervals. The culture medium after fermentation was centrifuged at 3000 rpm for 15 min to remove the insoluble substrates and cells. Supernatants were used as crude enzyme solution.

Enzyme assays

Carboxymethyl cellulase (CMC) activity (endo-1,4glucanase activity) was assayed by incubating (at 50°C for 30 min) 1 ml of 0.5% (W/V) CMC diluted in 0.2 mol/L sodium acetate (NaAc) buffer at pH 4.8 mixed with 1 ml of the enzyme solution (suitably diluted). The reducing sugars produced during incubation were determined following the DNS method by measuring the absorbance at 530 nm after adding 2 ml of the DNS reagent, heating in a boiling water bath for 5 min and cooling immediately.

The β-glucosidase activity was assayed by incubating (at 50°C for 30 min) 1 ml of 0.5 % (W/V) salicin diluted in 0.02 mol/L NaAc buffer at pH 4.8 mixed with 1 ml of the enzyme solution (suitably diluted). The reducing sugars produced during incubation were assayed as in the method previously described.

For enzyme activity on filter paper, 50 mg Whatman No.1 filter paper, 1 ml of 0.02 mol/L NaAc buffer (pH 4.8) and 1 ml of the enzyme solution (suitably diluted) were mixed together into a tube. The samples were incubated for 1 h at 50°C by the method of Eriksson et al. (1990); reducing sugars produced during incubation were assayed as in the method previously described.

For enzyme activity on cotton, 50 mg absorbent cotton, 1 ml of 0.02 mol/L NaAc buffer (pH 4.8) and 1 ml of the enzyme solution (suitably diluted) were mixed together into a tube. The samples were incubated for 24 h at 50°C according to the method of Vallander and Eriksson (1985) reducing sugars produced during incubation were assayed as in the method previously described.

The microcrystalline cellulase activity was assayed by incubating (at 50 °C for 2 h) 1 ml of 2% (W/V) microcrystalline cellulose diluted in 0.02 mol/L NaAc buffer at pH 4.8 mixed with 1mL of enzyme solution (suitably diluted) according to the method of Coudray et al. (1982). The samples were centrifuged at 5000 rpm for 10 min to remove insoluble substrates. Reducing sugar in the supernatant was assayed following the DNS method. One unit of enzyme activity is defined as the amounts of enzyme that liberated 1 µg of glucose in 1 min at 50°C.

Preparation of endocellular enzyme and cell wall/envelope enzyme

A loopful of slant culture was inoculated in 150-ml Erlenmeyer flask containing 30 ml beef extract-peptone liquid medium (3 g beef extract, 10 g peptone and 5 g NaCl per liter) at pH 7.0 to 7.2. The flasks were incubated at 30°C on a gyratory shaker at 170 rpm for 10 h. The culture was used as inoculum. Medium with the following composition: 3 g beef extract, 10 g peptone, 5 g NaCl, 5 g glucose, 2 g $NaNO_3$ at pH 7.0 to 7.2 was utilized for endocellular enzyme and cell wall/envelope enzyme. Two millliter (2 ml) of inoculum was inoculated in a 500-mL Erlenmeyer flask containing 200 ml cultivation medium. The flasks were incubated at 30°C on a gyratory shaker at 170 rpm for 10 h (*Bacillus* sp. X10-1-2) and 18 h (*Bacillus* sp. X18), respectively. The culture medium after fermentation was centrifuged at 1000 g for 10 min to collect cells. The cells were washed once, cooled with 0.05 mol/L phosphoric acid buffer (pH 7.2) and centrifuged. The pellets were re-suspended in chilled 0.05 mol/L phosphoric acid buffer (pH 7.2) at a ratio of 1:10 (10 ml buffer per gram wet cell weight). The suspension was disrupted by probe sonication 30 times for 30 s at 500 won ice. The sonicated suspension was centrifuged at 1000 g for 10 min at 4°C to remove undisrupted cells. The particulate fraction called cell wall enzyme (or cell envelope enzyme associated) was obtained as a pellet by centrifugation at 20000 g for 20 min at 4°C. The supernatant formed cytosolic fraction called endocellular enzyme. The pellet was suspended in 0.05 mol/L phosphoric acid buffer (pH 7.2). The cell wall enzyme (or cell envelope enzyme associated) and endocellular enzyme were dialyzed against deionized water at 4°C for 12 h. To prevent the contamination with bacteria, 0.02% sodium azide was added to the deionized water. The dialyzate was frozen for the following assays.

The reactions of endocellular enzyme and cell wall/envelope enzyme with inducers

The enzyme solution and sugar solution were prepared with deionized water. The initial enzyme solution and sugar solution were 20 mg/ml. The reaction mixtures, mixed at a ratio of 1:1 of the initial enzyme solution and sugar solution in 1.0 ml Eppendorf tubes, were incubated at 37°C for 24 h, and subsequently were centrifuged at 16000 rpm for 30 min at 0°C. The supernatants were collected for HPLC analysis. The experiment was repeated three times for each condition and the average of the three trials was calculated. The relative standard deviation (RSD) using this method was approximately 5%.

HPLC analysis

Products in reaction of endocellular enzyme and cell wall/envelope enzyme on inducers were analyzed on a Waters analytical HPLC instrument with refractive index detector. A 15 µl of the samples were injected into a carbohydrate column (4.6 ×250 mm). Acetonitrile and water (75/25; V/V) were pre-mixed, degassed, filtered and used as the mobile phase for analyses at a flow rate of 1.4 ml/min.

RESULTS

The results in Table 1 indicate that the species of cellulases (CMCase, β-glucosidase, Fpase, microcrystalline cellulose, activities on cotton) produced by the two strains of *Bacillus* sp. were same, though their activities differed from each other. For example, CMCase, β-glucosidase, Fpase, microcrystalline cellulose, and activity on cotton were 2.71, 3.83, 12.93, 1.50 and 17.99 IU/ml produced by *Bacillus* sp. X10-1-2 using sorbose as carbon source, respectively while cellulase activities of *Bacillus* sp. X18 were 1.17, 4.35, 2.62, 0 and 9.43 IU/ml, respectively. All

Table 1. Cellulase activities produced by *Bacillus* sp. X10-1-2 and *Bacillus* sp. X18 in the presence of different carbon sources (IU/ml).

Carbon source	Cellulase activity of *Bacillus* sp. X10-1-2					Cellulase activity of *Bacillus* sp. X18				
	CMCase	β-Glucosidase	Fpase	Microcrystalline cellulase	Activity on cotton	CMCase	β-Glucosidase	Fpase	Microcrystalline cellulase	Activity on cotton
No carbon source	nd	0.01	nd	nd	nd	nd	0.09	2.52	nd	2.11
CMC	nd	0.02	nd	nd	nd	nd	nd	nd	nd	nd
A	nd	0.04	nd	nd	nd	nd	nd	nd	nd	nd
B	nd	0.04	nd	nd	nd	nd	0.01	nd	nd	nd
C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
D	nd	0.00	nd	nd	nd	nd	0.06	nd	nd	nd
CMC+E	2.34	6.07	12.15	nd	10.60	3.93	3.05	9.51	nd	5.70
A+E	nd	2.63	11.43	0.02	13.06	3.28	3.70	9.64	nd	5.12
B+E	0.56	3.90	11.09	nd	14.84	2.38	1.25	11.23	nd	3.50
C+E	0.52	5.49	10.83	nd	15.45	2.74	1.48	9.48	nd	4.11
D+E	5.14	2.87	13.93	nd	20.33	3.77	4.73	4.00	nd	12.42
CMC+F	nd	2.90	14.49	0.36	14.07	1.34	2.02	3.49	nd	8.18
A+F	nd	2.53	14.20	nd	13.84	1.73	3.19	4.11	nd	7.16
B+F	nd	2.42	12.73	nd	12.27	2.89	4.87	4.20	nd	8.95
C+F	1.64	3.29	14.83	nd	16.49	2.81	2.82	2.73	nd	9.17
D+F	2.52	2.49	14.18	nd	16.89	2.57	3.76	4.00	nd	14.84
Glucose	nd	1.85	15.14	nd	16.51	2.18	21.46	4.56	nd	5.59
Cellobiose	nd	0.88	13.38	nd	14.03	2.04	3.56	4.28	nd	11.20
Fructose	4.02	3.61	13.79	0.18	16.41	3.88	6.37	4.10	nd	6.42
Maltose	1.61	2.19	12.66	0.82	11.07	2.94	4.62	4.05	nd	9.75
Sorbose	2.71	3.83	12.93	1.50	17.99	1.17	4.35	2.62	nd	9.43
Lactose	nd	1.19	12.75	0.08	14.41	2.24	3.15	4.06	nd	11.03
Xylose	1.81	2.75	11.91	0.89	20.98	1.13	2.91	3.27	nd	15.84
Galactose	1.66	2.14	12.53	nd	16.26	2.30	3.13	4.08	nd	12.75
Arabinose	1.22	0.75	13.29	nd	16.21	0.78	0.90	3.74	nd	21.04
Mannose	5.34	5.92	10.96	0.07	24.12	6.47	7.76	7.34	nd	27.66
Rhamnose	3.41	4.26	6.89	0.01	19.48	5.83	8.00	8.10	0.49	32.36
Soluble starch	nd	1.05	0.37	nd	0.01	nd	0.51	3.44	1.08	4.55
Sucrose	nd	nd	nd	nd	nd	nd	0.05	0.26	1.01	1.57
Reffinose	nd	0.00	nd	nd	nd	nd	0.05	nd	nd	nd
Xylan	nd	0.07	nd	nd	nd	nd	0.07	nd	nd	nd
Mannitol	nd	0.01	nd	nd	nd	nd	0.10	nd	nd	nd
Sorbitol	nd	0.03	nd	nd	nd	nd	0.03	nd	nd	nd
Xylitol	nd	0.00	nd	nd	nd	nd	0.03	nd	nd	nd

A: Microcrystalline cellulose; B: Filter paper; C: Cotton; D: Sigmacell; E: glucose; F: Cellobiose. nd: Not detected.

cellulases showed activities on salicin, filter paper and cotton, no matter which inducer or which strain of *Bacillus* sp. was utilized. The crude enzyme could react on salicin, which means that there was β-glucosidase activity in it, while there were also exo- and endo- synergetic effect in fermented broth, as crude enzyme could degrade filter paper and cotton. When exo-cellulase solely reacted on microcrystalline cellulose, only a little reducing sugar was released, while in this study, the activity of cellulase on microcrystalline cellulose was defined by using the quantity of reducing sugar, thus this activity was very hard to be detected (Desphande et al., 1984). It can be concluded that all the conditions that could induce cellulase production may accelerate main cellulases com-ponents synthesizing at a certain rate. This showed that the synthesis and expression of all cellulase components were synergic. This conclusion supported the work of Wang (2002).

The data in Table1 (cellulase activities produced by *Bacillus* sp. X10-1-2 and *Bacillus* sp. X18 under the condition of different carbon sources) were both the results of 24 h, as all cellulase activities appeared to reach maximum value in 24 h. Results (Table 1) show that bacteria would quickly produce cellulase by easy metabolizable carbon sources as inducer.

As we know, cellulase is an inducible enzyme (Hohn and Sahm, 1983). In the process of its production by means of microorganism fermentation, the substrates are not only used as carbon sources, but also the main sources of inducer. In order to make sure that there is a real inducer for the two strains of *Bacillus* sp., several common cellulose materials (such as CMC, microcrystalline cellulose, filter paper, cotton, sigmacell) and soluble saccharide (glucose, cellobiose, fructose, maltose, sorbose, lactose, xylose, galactose, arabinose, mannose, rhamnose, soluble starch, reffinose, xylan, mannitol, sorbitol, xylitol) were chosen as carbon sources. Catabolite repression plays an important role in the regulation and secretion of inducible enzyme. Such repression effect has been observed in other organisms (Magnelli and Forchiassin, 1999; Beguin, 1990; Hrmova et al., 1991). To prevent catabolite repression, 0.1% (W/V) of lower initial concentration of soluble saccharide in the fermentation medium was used. The results in Table 1 indicate that when pure cellulose, whether crystal (microcrystalline cellulose, filter paper, cotton, sigmacell) or non-crystal (CMC), soluble (CMC) or insoluble (microcrystalline cellulose, filter paper, cotton, sigmacell), was used as the sole carbon source, except for faint β-glucosidase (0.01~0.06 IU/ml) these two strains of *Bacillus* sp. could not be induced to produce cellulase. Induction mechanism for their production of cellulase is quite different from that of fungi. Natural cellulose materials are always good inducible carbon sources for fungi. In this research, only soluble carbon sources with reducing group (glucose, cellobiose, fructose, maltose, sorbose, xylose, galactose, arabinose, mannose, and rhamnose) could induce them to produce cellulase (highest cellulase activity was reached at 32.36 IU/ml, this was activity on cotton produced by *Bacillus* sp. X18 using rhamnose as carbon source). When soluble non-reducing sugars such as xylan, sucrose, etc, were utilized as sole carbon sources, cellulase activity could not be detected in fermented broth. However, some reducing sugars would not be induced to produce cellulase after their reducing groups were substituted by hydroxyl groups to become sugar alcohol like mannitol, sorbitol, and xylitol.

The results of Figure 5 and Table 2 show that the retention times of products formed in the reactions of endocellular enzyme and cell wall/envelope enzyme with inducers were almost the same as standards (for example, retention time of xylose standard on HPLC was 4.07 min. Retention time of products formed in the reaction of endoenzyme and cell wall/envelope enzyme of *Bacillus* sp. X10-1-2 and *Bacillus* sp. X18 were 4.07 and 4.08 min, respectively). This illuminated that enzyme located on cell wall/envelop enzyme and endoenzyme was inactive on inducers that could induce *Bacillus* sp. to produce cellulases. Thus, these small molecules inducers might be regarded to enter cells directly and serve the function of induction. Induction mechanism of cellulase production in two *Bacillus* sp. was quite different from that of fungi.

DISCUSSION

According to the results, a conclusion might be drawn that these two strains of *Bacillus* sp. have similar inductive chart. It means that their induction mechanisms seem alike. Eberhart and Beck (1973) have suggested a quasi-regulatory role of cell wall bound β-glucosidase in enzyme regulation by alteration or destruction inducer molecules. However, it is not clear if the products formed in the action of cell wall bound enzyme and endoenzyme after these small molecules saccharide that can induce *Bacillus* species to produce cellulase enter cells is same or not, that is, real inducer is the same or not, or these inducer enter cells directly to serve function of induction without any transformation. So, the endoenzyme and cell wall/envelope enzyme from two *Bacillus* sp. were prepared, which were reacted with inducer for cellulase, then qualitative analysis of the products was developed to confirm the accurate one. These two *Bacillus* spp. may be simultaneously affirmative and can only distinguish molecules with certain structural groups. These molecules were transformed to induce cellulase by some alteration or destruction on cell envelope or in cells. On the other hand, all the small molecular soluble sugar might enter into cells directly, but only a kind of molecule with specific structural groups can bind with certain protein, then, this protein directly or indirectly affects some kind of protein bound with DNA; the transcription and expression of cellulase gene is then activated.

Since cellulose and the cellulosic components in lingo-cellulosic biomass substrates are essential for the forma-

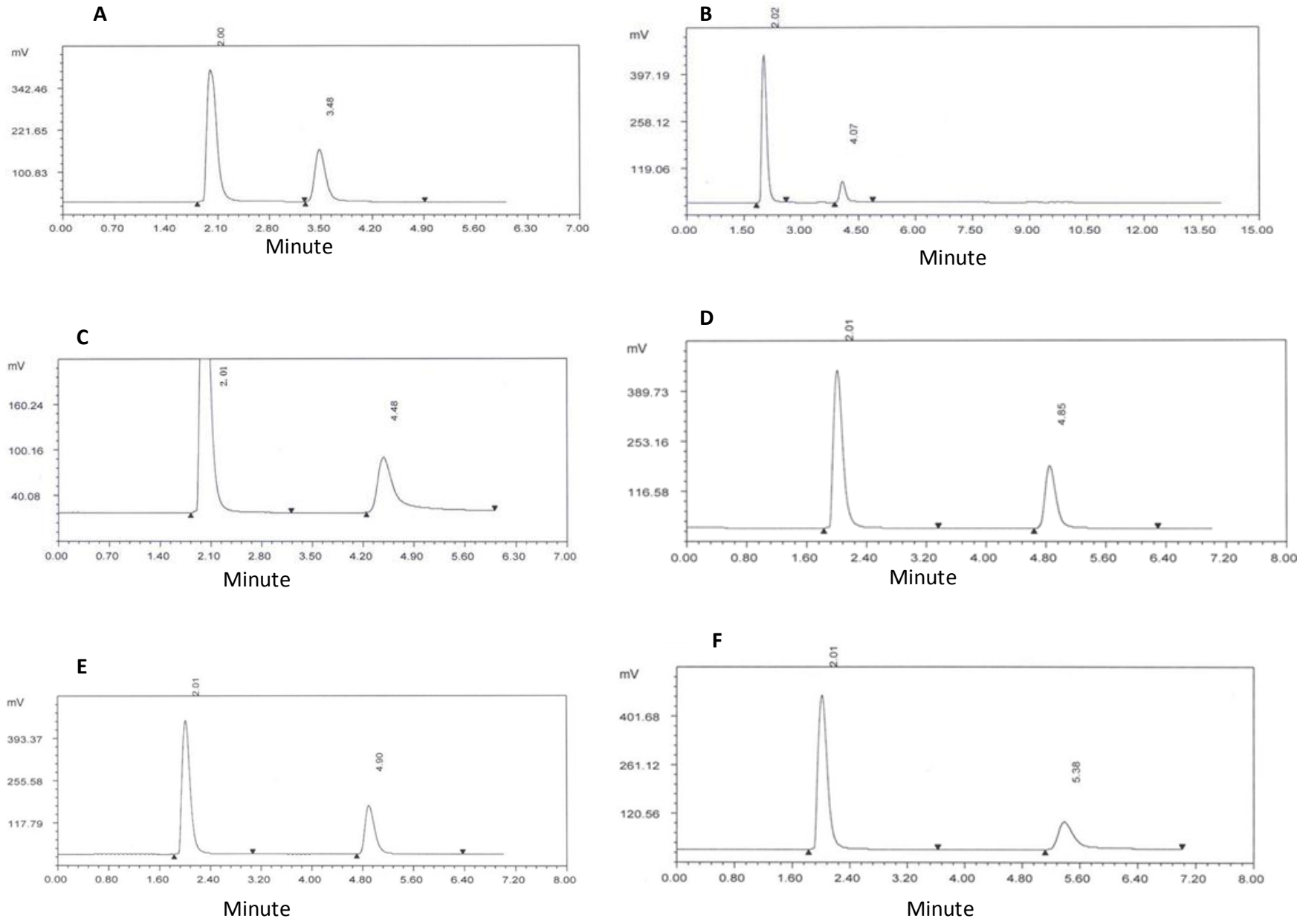

Figure 5. HPLC graph of sugar standards: (a) rhamnose (b) xylose (c) arabinose (d) sorbose (e) fructose (f) mannose (g) glucose (h) galactose (i) cellobiose (j) maltose (k) lactose.

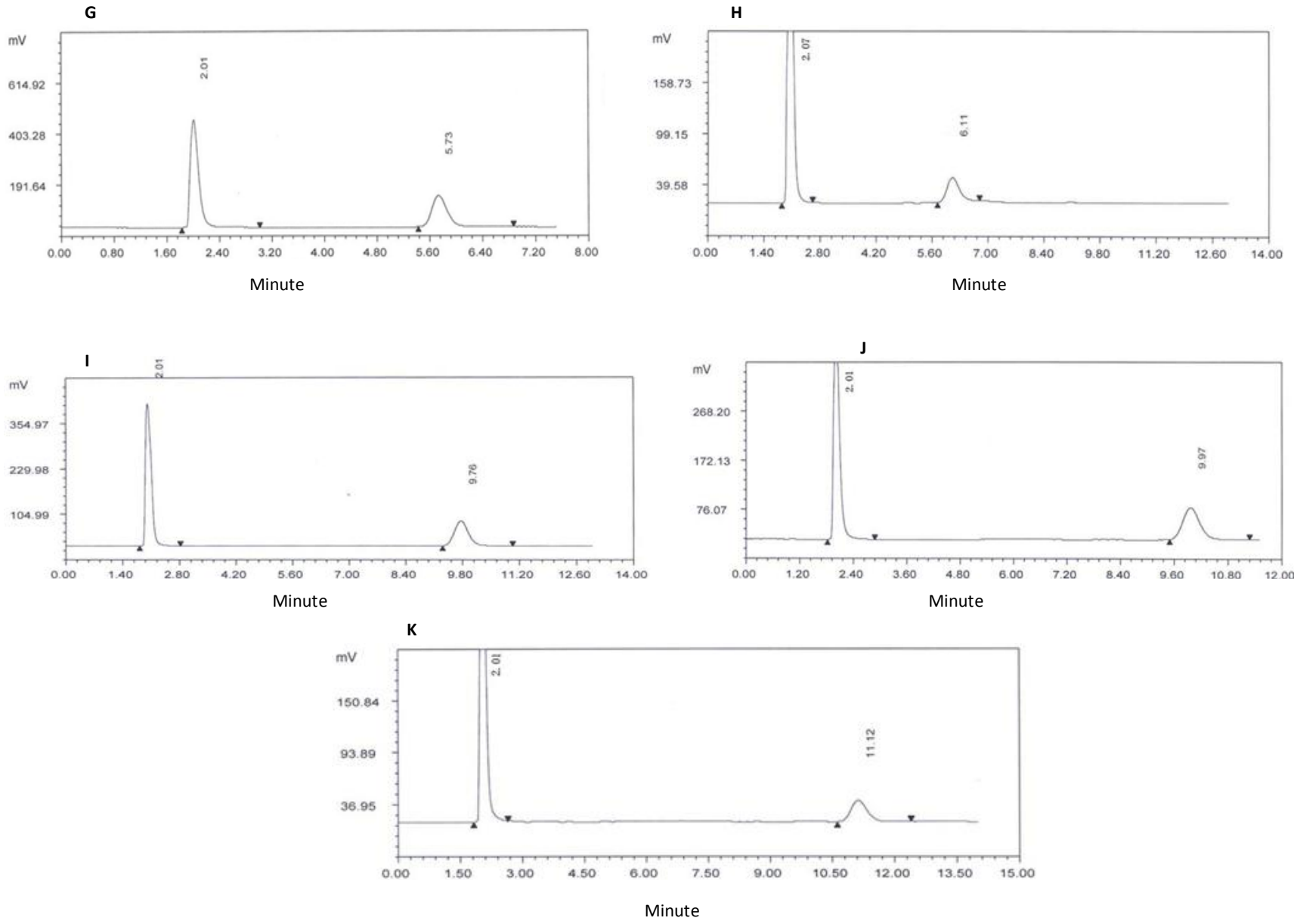

Figure 5. Contd.

Table 2. Retention time of samples on HPLC (min).

Number	Sugar standard	A	B	C	D
1	3.48	3.50	3.50	3.50	3.50
2	4.07	4.07	4.08	4.07	4.08
3	4.48	4.51	4.51	4.49	4.49
4	4.85	4.87	4.86	4.84	4.84
5	4.90	4.95	4.93	4.93	4.91
6	5.38	5.43	5.39	5.38	5.38
7	5.73	5.77	5.73	5.73	5.71
8	6.11	6.17	6.11	6.10	6.10
9	9.76	9.78	9.78	9.74	9.74
10	9.97	9.95	9.95	9.95	9.95
11	11.12	11.12	11.12	11.12	11.12

A: Retention time of products formed in the reaction of endoenzyme of *Bacillus* sp. X10-1-2 with different sugar; B: Retention time of products formed in the reaction of cell wall/ envelope enzyme of X10-1-2 with different sugar; C: Retention time of products formed in the reaction of endoenzyme of *Bacillus* sp. X18 with different sugar; D: Retention time of products formed in the reaction of cell wall/ envelope enzyme of *Bacillus* sp. X18 with different sugar.

tion of mRNA to support maximum formation of cellulases at the transcription level (Gutierrez-Nova et al., 2003), according to the stated analysis, a hypothesis might be drawn upon the mechanism of the two *Bacillus* spp. producing cellulases. Natural cellulose castoff, like straw, wheat bran, and so on, most possibly contained a small quantity of reducing sugar which could induce *Bacillus* sp. to produce cellulases. Therefore, when *Bacillus* species was used to degrade natural cellulose materials under the natural conditions, the reducing sugar entered the cells of these bacteria and induced them to produce cellulases, and then the cellulases were released out of cells to degrade cellulose. In fact, several reports in the literature suggested that wheat bran was demonstrated as the best carbon source to cellulase production in many organisms (Jiang and Liu, 1999; Chen and Qu, 2000; Hanif et al., 2004). On the other hand, natural cellulose often included some associated components like lignin and hemicellulose; hemicellullose is a kind of in homogeneous polymer whose degree of polymerization is less than 200, composed of xylose, galactose, mannose, arabinose, glucose and glycuronate, and they physically combine with cellulose. *Bacillus* sp. might produce a basic level or a constitutive amount of cellulase or other substances that could degrade hemicellulose to soluble hydrolysis products like xylose, galactose, mannose, etc., that could function as an inducer; these products entered the cells inducing *Bacillus* sp. to produce cellulases. Furthermore, cellulolytic microorganisms can be found in all biota where cellulosic waste accumulates. They usually occur in mixed populations comprising cellulolytic and non-cellulolytic species, which often interact synergistically. Non-cellulolytic species might provide cellulolytic species with inducers formed in the reaction of non-cellulolytic species with non-cellulose components in cellulosic waste accumulates. These interactions lead to the complete degradation of cellulose.

Conclusions

The study presents that the induction of cellulase production in two *Bacillus* sp. were investigated by means of measuring cellulase activities under the condition of different carbon sources. The results indicate that cellulase could not be induced by cellulose material as a sole carbon source. Instead, they could be induced by monosaccharide or disaccharide with reducing group. Moreover, the expression of cellulase components was synergistic.

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Antimicrobial activities of the leaves and roots of *Elaeagnus umbellata* Thunb

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Plants are the rich natural source of bioactive compounds. The more diversified composition of the plants makes their role as biomedicine. These bioactive molecules are often lethal to both plants and animals. Based on ethnomedical use, the leaves and root of *Elaeagnus umbellata* (Elaeagnaceae) were extracted successively with various organic solvents and water. These crude extracts were screened for their toxic potential against three Gram- positive bacteria, five Gram- negative bacteria, one yeast, and one fungus by using disc diffusion method. The acetone, petroleum ether, ethyl acetate, chloroform, ethanol and methanol extracts of the plant possessed significant antimicrobial activities on both Gram- positive and Gram- negative bacteria. The acetone, petroleum ether, ethyl acetate and methanol extracts of leaves and roots of the plant exhibited prominent activities while chloroform, ethanol extracts showed moderate activity and water extract showed no activity against all the tested bacteria. Ethanolic and methanolic extracts also showed considerable activity against fungus and yeast. The root extracts of the plant were found more active against the microorganisms.

Key words: *Elaeagnus umbellata,* extracts, fungi, yeast, antibiotic discs.

INTRODUCTION

Biological screening is an important step in the evaluation of medicinal plants activity (Nisar et al*.,* 2011; Qayum et al., 2012). Thus, any phytochemical investigation of a given plant will reveal a spectrum of its bioactive chemical constituents. Natural products represent virtually inexhaustible reservoir of molecules, most of which are hardly explored and could constitute lead molecules for new antimalarial drugs, such as artemisinin, isolated from *Artemisia annua* (Kayser et al., 2003). Historically, pharmacological screening of compounds of natural or synthetic origin has been the source of innumerable therapeutic agents. Random screening as tool in discovering new biologically active molecules has been most productive in the area of antibiotics (Gerhartz et al., 1985; Kroschwitz, and Howe-Grant, 1992). Even now, contrary to common belief, drugs from higher plants continue to occupy an important niche in modern medicine.

On a global basis, at least 130 drugs, all single chemical entities extracted from higher plants, or modified further synthetically, are currently in use, though some of them are now being made synthetically for economic reasons (Newman et al*.,* 2000). In present time, multiple drug resistance in microbial pathogens become a serious health problem to humankind worldwide (Peng et al., 2006). It is aroused due to indiscriminate and repetitive use of antimicrobial drugs (Shariff, 2001). Synthetic drugs are not only expensive and inadequate for the treatment of diseases but also often associated with adulterations and side effects. Therefore, there is need to search new infection fighting strategies to control microbial infections. Due to the same reason, during the past decade, traditional systems of medicines have become increasingly important in view of their safety (Krishnaraju et al., 2006) and research

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is carried out in order to determine antimicrobial potential of medicinal plants. Bioassay has been used successfully to monitor the isolation of cytotoxic, antimalarial, insecticidal and antifeedent (Siqueira et al., 1998; Perez et al., 1997; Oberlies et al., 1998; Labbe et al., 1993).

Medicinal plants represent a rich source of antimicrobial agents. Plants are used medicinally in different countries and are a source of many potent and powerful drugs (Srivastava et al*.,* 1996). A wide range of medicinal plant parts is used for extracts as raw drugs and they possess varied medicinal properties. The different parts used include root, stem, flower, fruit, twigs exudates and modified plant organs. While some of these raw drugs are collected in smaller quantities by the local communities and folk healers for local use, many other raw drugs are collected in larger quantities and traded in the market as the raw material for many herbal industries (Uniyal et al*.*, 2006).

Although hundreds of plant species have been tested for antimicrobial properties, the vast majority of them have not been adequately evaluated (Balandrin et al*.*, 1985). Medicinal plants have been relied upon by 80% of the world population for their basic health care needs. Pakistan is no exception, as it has a variety of plants of medicinal importance (Tareen et al*.,* 2002). The herbs are extensively used for treating diseases, however their commercial exploitation is limited due to the lack of scientific knowledge for their use (Ahmad et al*.,* 2003).

Among these plants, *Elaeagnus umbellata* Thunb*,* also called cardinal olive, autumn olive or autumn Elaeagnus (Dirr*,* 1983), a wild shrub belonging to the family Elaeagnaceae, is native to China, Japan and Korea, and is also found in Afghanistan and India (Potter*,*1995). The plant was introduced to the US in the 1830s from East Asia as an ornamental plant (Dirr*,* 1983).

E. umbellata is widely distributed at a height of 5000-7500 feet above sea level in Muzaffarabad Azad Kashmir. It is abundantly found in Himalayan regions of Pakistan (Hensley, 1984; Ahmad et al*.,* 2005). The *E. umbellata* is a large spreading, spiny-branched shrub often obtaining 3.5-5.5 m in height, and 3.5-5.5 mm in width. The foliage is light green on top and a silvery green on the bottom. Leaves are alternate and petiolated in small lateral clusters on twigs (Eckardt, 1987).

The fruit / berries are silvery with brown scales when immature and ripen to a speckled red in September - October (Sternberg, 1982). Its berry is an excellent source of vitamins A, C, E, flavonoids, essential fatty acids (Chopra et al*.,* 1986), lycopene, carotene, lutein, phytofluene and phytoene. The lycopene content of the *E. umbellata* fruit is 17 times greater than that of tomato (Kohlmeier et al*.,* 1997; Fordham et al., 2001). Many studies have proved that lycopene protects against myocardial infarction (Kohlmeier et al*.,* 1997) and various forms of cancers including prostate cancer (Clinton, 1998; Giovannucci et al*.,* 1995). The seeds of the plant are used as a stimulant in the treatment of coughs and seed oil is used in the treatment of pulmonary affections (Chopra et al*.,* 1986). Various phytochemicals including palmitic acid (16.9%), eugenol (11.1%), methyl palmitate (10.5%), 4-methyl anisole (33-42.7%) and 4-methyl phenol (10.9-13.3%) have been isolated from the flowers of the plant (Matthews, 1994).

The extracts of the plant and its chemical constituents exhibit antimicrobial properties, which may be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency (Nabeela and Zaheer, 2003).

Many plants have been used due to their antimicrobial traits, which are due to the compounds synthesized in the secondary metabolism processes, that is, phenolics and tannins. *E. umbellata* is one of such plants which are being used against infectious diseases. Although antibacterial activity of the aerial parts of the plant had been studied by Sabir et al. (2007) against four bacteria, a detailed antimicrobial potential of aerial and ground parts of *E. umbellata* has not been studied, the *in vitro* antimicrobial activity of the leaves and roots of the plant growing wild in Azad Jammu and Kashmir was evaluated by using disc diffusion method against eight bacteria, one fungus and one yeast. The present work appears to be the first detailed antimicrobial bioassay report on aerial as well as ground part of the plant.

MATERIALS AND METHODS

Fresh plant parts were collected randomly from different localities of Muzaffarabad, Azad Jammu and Kashmir, Pakistan. The plant was identified by a Senior Botanist at the Department of Botany, where a voucher specimen (No. Bot. UAJK 1021) is deposited. The collected plant parts were separately air dried under shade and then homogenized to fine powder and finally stored in air tight bottles at 4°C.

Aqueous extracts

Forty-five gram (45 g) of each ground plant part material was extracted with distilled water in soxhlet extraction apparatus (Thomas, 1977). These extracts were collected separately and each extract was dried on rotary evaporator under reduced pressure. The last traces of the water were evaporated at water bath, which was used as a source of heat (Rawlins and Tindall, 1977).

Organic solvent extraction

A portion (25 g) of each dried powdered plant material was soaked separately in 250 ml petroleum ether, acetone, ethyl acetate, chloroform, ethanol and methanol for ten days at room temperature (25±2°C). The solvents extracted material was filtered in flasks (Rawlins and Tindall, 1977). The extracts were then filtered through Whatman filter paper No.1. All organic extracts were dried on a rotary evaporator under reduced pressure, weighed and stored at 4°C till further analysis. .

Preparation of dilution

The dried aqueous, methanol, ethanol, petroleum ether, acetone,

ethyl acetate and chloroform extracts were then dissolved in their respective solvents in a proportion of 10 mg/ml. The concentration of reference antibiotics, that is, ciprofloxacin was 100 µg/ml and nystatin 1500 u/ml. *aureus, Bacillus subtilis, Enterococcus faecium,* five Gram-negative bacteria, *Escherichia coli*, *Bordetella bronchisiptica, Salmonella typhi, Pseudomonas aeruginosa, Pseudomonas syringae* (local isolate)*,* one yeast *Saccharomyces cerevisiae* (local isolate) and one fungus *Aspergillus flavus* (local isolate)*,* were used to check the antimicrobial potential of different extracts of the selected plant parts.

The pure bacterial, fungal and yeast strains were obtained from the Department of Pathology Muzaffarabad Medical College Teaching Hospital, Muzaffarabad Azad Jammu and Kashmir. Bacterial strains were cultured overnight at 37°C in nutrient agar (NA, Oxoid, Hampshire, UK) while fungal strains were cultured overnight at 28°C using sabouraud's dextrose agar (SDA, Oxoid, Hampshire, UK).

Antimicrobial assay

The antimicrobial activity was determined by disc diffusion method (Vander and Vlientnck, 1991). Briefly, 100 µl of suspension of tested microorganisms, containing 10^8 colony-forming units (cfu)/ml of bacteria cells and 10^5spores/ml of fungi was spread on sterilized nutrient agar (NA) and SDA medium, respectively. The disc (6 mm in diameter) was individually impregnated with extract samples, placed on the agar plates which had previously been inoculated with the tested microorganisms. A disc without compound was used as a negative control. In the second series of experiment, antibiotic discs prepared from the dilution of commercially available standard reference antibiotics, that is, ciprofloxacin and nystatin were placed on top of the medium in the center of Petri dishes following the disc diffusion method (Vander and Vlientnck, 1991).

The purpose of this experimental set was to compare the antimicrobial activity of the standard reference antibiotics with that of the solvent extracts of leaves and roots of *E. umbellata*. Plates, after 2 h at 4°C, were incubated at 37°C for 24 h for bacteria and at 28°C for 72 h for fungal strains in incubator (Synou, Germany). Antimicrobial activity was evaluated by measuring the diameter of the growth inhibition zones by zone reader (MAS GmbH, Germany) in millimeters for the organisms and comparing to the controls (Rehman et al., 2001).

Statistical analysis

All values were expressed as means ± standard error. The data for each microorganism were analyzed by using one way analysis of variance (ANOVA) technique and means were compared by using least significant difference (LSD) at 5% (0.05) probability level (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Plant extracts are being studied against bacteria for years and in a more intensified way in the last three decades. During this period, alot of antimicrobial screening evaluations has been published based on the traditional use of Chinese, African and Asian plant drugs (Forestiere et al*.,* 1988). In the present study, the antimicrobial activities of acetone, petroleum ether, ethyl acetate, chloroform, ethanol, methanol and water extracts of *E. umbellata* leaves and roots were determined. The results of the antimicrobial screening of different solvents extracts of leaves and root of *E. umbellata* against 8 bacteria, 1 yeast and 1 fungus are presented in Tables 1, Figure 1 and Table 2, Figure 2.

According to previous investigations of Sabir et al*.* (2007), the samples prepared from the aerial parts of *E. umbellata* specie growing in Rawalakot Azad Jammu and Kashmir showed antimicrobial activity against four bacteria tested. They studied antibacterial activity of acetone, chloroform, methanol and ethanol extracts of leaves along with various extracts of berries and flowers against four bacteria namely *E. coli, P. aeruginosa, S. aureus* and *B. subtilis*. The present study shows 50 to 100% better results than the previous antibacterial activity. The acetone extracts of leaves showed antimi-crobial activity against *S. aureus, B. subtilis, E. coli, B. bronchisiptica,* while root extract exhibited activity against *S. aureus, B. subtilis, E. faecium, E. coli, B. bronchisiptica, P. aeruginosa, P. syringae,* and *S. typhae.* The results are presented in Tables 1, Figure 1 and Table 2 and Figure 2.

The petroleum ether extract of the leaves of *E. umbellata* exhibited antimicrobial activities against *S. aureus E. coli and B. bronchisiptic* while root extract showed remarkable activity against *S. aureus, B. subtilis, E. faecium, E. coli, B. bronchisiptica, P. aeruginosa, P. syringae* and *S. typhae.* The results are presented in Table 1, Figure 1 and Table 2, Figure 2.

The ethyl acetate extract of the leaves of the plant showed high antibacterial activity against *S. aureus, B. subtilis, E. coli, B. bronchisiptica, P. aeruginosa,* and *P. syringae* while the extract of root also showed promising activity against *S. aureus, B. subtilis, E. faecium, E. coli, B. bronchisiptica, P. aeruginosa, P. syringae,* and *S. typhae* (Table 1, Figure 1 and Table 2, Figure 2). The chloroform extract of the leaves showed activity against *E. coli* (20.56±0.23 mm), *P. aeruginosa* (15.00 ±0.00 mm), *P. syringae* (17.16 ±0.16 mm), *S. aureus,* (10.00 ±0.00 mm) *B. subtilis* (16.22 ±0.08 mm)*, B. bronchisiptica* (16.30 ±0.16 mm)*,* while *S. typhae, E. faecium, S. cerevisiae* and *A. flavus* were found inactive (Table 1, Figure 1). The chloroform extract of the root of *E. umbellata* exhibited moderate activity against all the tested microorganisms used except *S. cerevisiae* and *A. flavus.* The mean diameter of zones of inhibition of the extract against *S. aureus, B. subtilis, E. faecium, E. coli, B. bronchisiptica, P. aeruginosa, P. syringa* and *S. typhae* were 10.00 ±0.00, 9.66 ±0.33, 9.66 ±0.33, 20.06 ±0.06, 10.06 ±0.06, 15.1 ±0.1, 9.66 ±0.33 and 9.66 ±0.33 mm, respectively (Table 2, Figure 2). The chloroform extracts showed comparatively more *in vitro* antimicrobial activity against bacteria as compared to the previous work on leaf extract by Sabir et al*.* (2007) which may be due to topographic variation on the chemical constituents of the plant*.*

The methanolic extracts of the leaves and root of the plant showed considerable activity not only against all tested bacteria but also exhibited considerable activity against the fungus and yeast. The zones of inhibition

Table 1. Antimicrobial activity profile of the leaves of *E. umbellata* Thunb.

S/N	Strain	Zones of inhibition (mm) ± standard error (S E M)								
		AC	PE	EA	CH	ET	MT	WT	CF	NS
1	*S. aureus*	19.35±0.15	14.5±0.05	20.33±0.17	10.00±0.00	12.9±0.35	16.16±0.08	0.00±0.00	32.13±0.13	0.00±0.00
2	*B. subtilis*	16.33±0.17	0.00±0.00	19.66±0.33	16.22±0.17	15.83±0.16	14.33±0.17	0.00±0.00	31.93±0.06	0.00±0.00
3	*E. faecium*	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	13.9±0.1	17.33±0.19	0.00±0.00	30.03±0.03	0.00±0.00
4	*E. coli*	19.22±0.11	16.11±0.10	22.96±0.23	20.56±0.23	17.46±0.33	16.33±0.16	0.00±0.00	31.83±0.17	0.00±0.00
5	*B. bronchisiptica*	16.93±0.06	20.33±0.15	20.83±0.17	16.30±0.16	14.9±0.2	17.19±0.16	0.00±0.00	31.5±0.1	0.00±0.00
6	*P. aeruginosa*	0.00±0.00	0.00±0.00	17.66±0.33	15.00±0.00	18.83±0.16	19.83±0.17	0.00±0.00	29.83±0.17	0.00±0.00
7	*P. syringae*	0.00±0.00	0.00±0.00	22.66±0.33	17.16±0.16	15.66±0.33	20.93±0.06	0.00±0.00	30.83±0.17	0.00±0.00
8	*S. typhae*	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	15.83±0.17	15.26±0.13	0.00±0.00	29.93±0.06	0.00±0.00
9	*S. cerevisiae*	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	12.66±0.06	14.82±0.18	0.00±0.00	0.00±0.00	21.00±0.33
10	*A. flavus*	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	10.23±0.1	09.33±0.17	0.00±0.00	0.00±0.00	15.00±0.26

AC, Acetone; PE, petroleum ether; EA, ethyl acetate; CH, chloroform; ET, ethanol ; MT, methanol; WT, water; CP, ciprofloxacin; NS, nystatin. Concentration of crude extracts, 100 mg/ml; ciprofloxacin, 100 µg/ml; nystatin, 1500 u/ml.

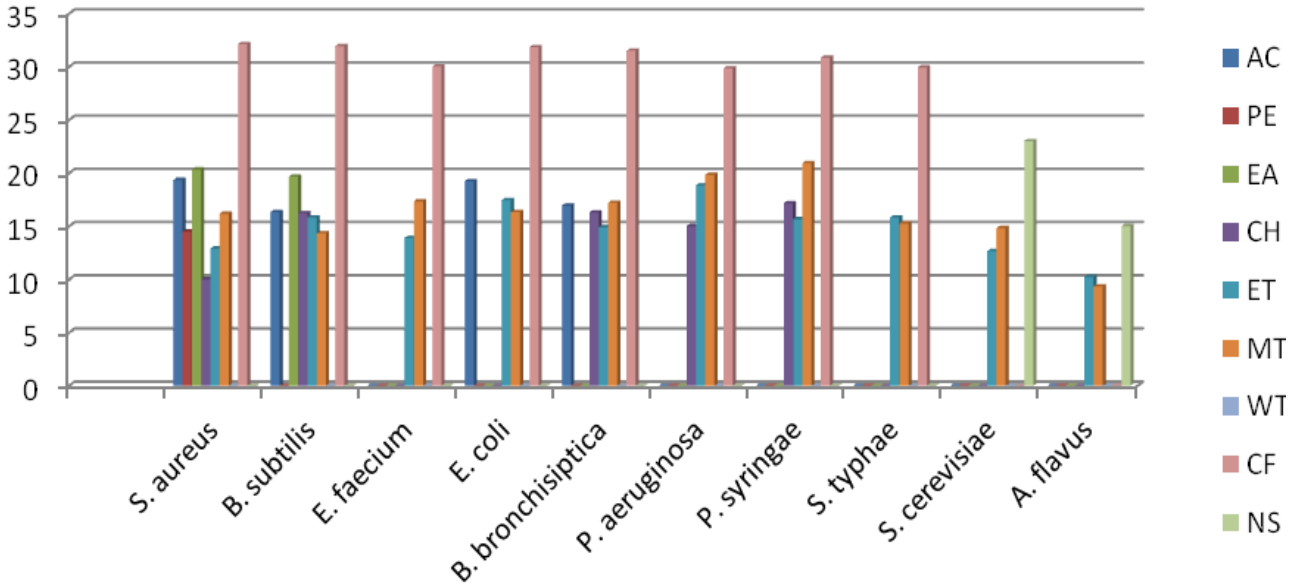

Figure 1. Antimicrobial activity of the leaves of *Elaeagnus umbellata.* AC: Acetone, PE: petroleum Ether, EA: ethyl acetate, CH: chloroform, ET: ethanol, MT: methanol, WT: water, CF: ciprofloxacin, NS: nystatin.

Table 2. Antimicrobial activity of the Roots of *E. umbellata* Thunb. with concentration of crude extracts of 100 mg/ml.

S/N	Microorganism	Zones of inhibition (mm) ± standard error (S E M)						
		Acetone	Petroleum ether	Ethyl acetate	Chloroform	Ethanol	Methanol	Water
1	*S. aureus*	17.83±0.17	19.9±0.1	14.83±0.17	10.00±0.00	10.67±0.33	20.24±0.06	0.00±0.00
2	*B. subtilis*	16.06±0.06	17.83±0.17	16.06±0.06	9.66±0.33	11.83±0.17	18.9±0.1	0.00±0.00
3	*E. faecium*	18.06±0.06	19.66±0.33	17.06±0.06	9.66±0.33	13.00±0.00	16.03±0.3	0.00±0.00
4	*E. coli*	20.83±0.17	20.06±0.06	19.83±0.17	20.06±0.06	15.83±0.16	24.33±0.17	0.00±0.00
5	*B. bronchisiptica*	13.06±0.03	16.1±0.01	16.06±0.03	10.06±0.06	10.66±0.33	18.66±0.15	0.00±0.00
6	*P. aeruginosa*	20.66±0.33	18.76±0.23	17.66±0.33	15.1±0.10	12.76±0.23	19.83±0.17	0.00±0.00
7	*P. syringae*	19.66±0.33	23.06±0.06	19.66±0.33	9.66±0.33	11.66±0.33	13.93±0.16	0.00±0.00
8	*S. typhae*	13.06±0.06	16.66±0.33	15.06±0.06	9.66±0.33	9.06±0.06	15.13±0.17	0.00±0.00
9	*S. cerevisiae*	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	14.11±0.1	15.22±0.16	0.00±0.00
10	*A. flavus*	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	11.75±0.15	10.77±0.13	0.00±0.00

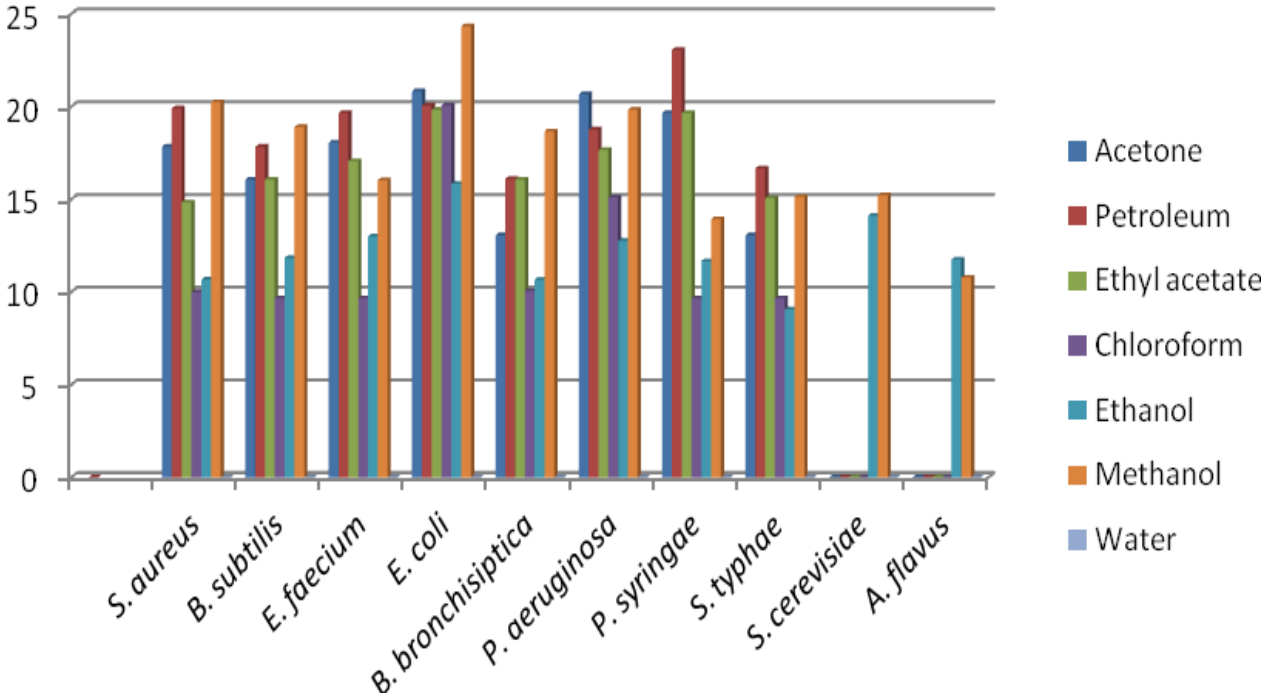

Figure 2. Antimicrobial activity of the roots of *Elaeagnus umbellata.*

produced by the leaves extract against *E. faecium, P. aeruginosa, P. syringae S. aureus, B. subtilis, E. coli, S. typhae and B. bronchisiptica* were 17.33 ± 0.19, 19.83 ± 0.17, 20.93 ± 0.06, 16.16 ± 0.08, 16.33 ± 0.16, 15.26 ± 0.13 and 17.19 ± 0.16 mm, respectively. The zone of inhibition produced by the methanolic extract of leaves against *S. cerevisiae and A. flavus* were found to be 14.82 ± 0.18 and 09.33 ± 0.17 mm, respectively. The zones of inhibition produced by the methanolic extract of root against *E. faecium, P. aeruginosa, P. syringae, S. aureus, B. subtilis, E. coli, S. typhae* and *B. bronchisiptica* were found to be 16.03 ± 0.3, 19.83 ± 0.17, 13.93 ± 0.16, 20.24 ± 0.06, 18.9 ± 0.1, 24.33 ± 0.16, 15.13 ± 0.17 and 18.66 ± 0.15 mm, respectively. The zones of inhibition against *S. cerevisiae* and *A. flavus* were 15.22 ± 0.16 and 10.77 ± 0.13 mm, respectively (Table 1, Figure 1 and Table 2, Figure 2).

Sabir et al. (2007) described that the ethanolic extract of the leaves of *E. umbellata* did not show any significant antimicrobial activity against *P. aeruginosa, S. aureus* and *E. coli* except *B. subtilis.* While in the present study, it was observed that the ethanolic extracts of the leaves and roots were found to be more active against Gram-positive and Gram-negative bacteria as well as against fungus and yeast. The ethanolic extract of the leaves showed antimicrobial activity against *E. coli* (17.46 ± 0.16 mm), *P. aeruginosa* (18.83 ± 0.06 mm), *P. syringae* (15.66 ± 0.33 mm), *S. aureus,* (12.90 ±0.35 mm), *B. subtilis* (15.83 ± 0.16 mm), *B. bronchisiptica* (14.09 ±0.2 mm), *S. typhae* (15.83 ±0.17 mm), *E. faecium,* (13.90 ±0.1 mm), *S. cerevisiae,* (12.66 ±0.06 mm) and *A. flavus,* (10.23 ±0.1 mm). The ethanolic extract of the root of *E. umbellata* also exhibited moderate activity against all the tested microorganisms including *S. cerevisiae* and *A. flavus.* The mean diameter of zones of inhibition of the extract against *S. aureus* (10.67 ±0.33 mm),, *B. subtilis* (11.83 ±0.17 mm), *E. faecium* (13.00 ±0.00 mm), *E. coli* (15.83 ±0.16 mm), *B. bronchisiptica* (10.66 ±0.33 mm), *P. aeruginosa* (12.76 ±0.23 mm), *P. syringae* (11.66 ±0.33 mm), *S. typhae* (9.06 ±0.06 mm), *S. cerevisiae* (14.11 ±0.1 mm) and *A. flavus* (11.75 ±0.15 mm), respectively. The detail results are shown in Table 1, Figure 1 and Table 2, Figure 2.

The water extracts of the leaves and roots represented no activity against the microorganisms (Table 1, Figure 1 and Table 2, Figure 2). The antibiotic, ciprofloxacin showed high activity against all the microorganisms used except *S. cerevisiae* and *A. flavus*. The activity profile against *S. aureus, B. subtilis, E. faecium, E. coli, B. bronchisiptica, P. aeruginosa, P. syringae, S. typhae* are presented in Table 1. The nystatin exhibited activity against the yeast and fungus while all the bacteria were resistant to nystatin (Table 1, Figure 1). Avato et al. (1997) reported that extracts of *Bellis perennis* have a high antimicrobial activity against bacteria than fungus. The results of Zavala et al. (1997) were similar to ours. They showed that extracts from some plants have high activity against bacteria than yeast and fungus. On the other hand the antimicrobial activity against Gram-negative bacteria was more effective than Gram-positive bacteria (Table 1, Figure 1 and Table 2, Figure 2).

The antibacterial and antifungal activity of the plant extracts may be due to the flavonoids and phenolics which have already been reported in the leaves of the plant (Chopra et al., 1986). These compounds are usually extracted in organic solvents. Actually, phenolics not only attack the cell wall and cell membrane, thereby destroying its permeability and releasing the intracellular constituents (ribose, sodium, glutamate, and so forth) but also interfere with membrane function, for example, electron transport chain, nutrient uptake, protein and nucleic acid synthesis, and also affect enzyme activity. The bioactive compounds might have several invasive targets that could lead to inhibition of the bacteria and fungi (Sabir et al., 2007). The results reveal that the extent of inhibition is variable for different extracts against different microbes. It seems very likely, therefore, that the antimicrobial compounds extracted from *E. umbellata* may inhibit bacteria and fungi by a different mechanism than that of currently used antibiotics and may have therapeutic value as an antimicrobial agent against multidrug resistant microbial strains. The method used by traditional healers for treating a bacterial and fungal infections, is administering a decoction of the plant or by using a part by boiling it in water. According to our results, an organic solvent extract is more effective in reducing microbial infections compared to water. We have recently isolated three coumarins, namely, 7-Hydroxy-chromen-2-one, 7, 8-Dihyroxychromen-2-one, 3-(2,2,3,4,5-Pentahydroxy-hexyloxy)-chromen-2-one and an anthraquinone from *E. umbellata* which showed partial suppression of parasitic growth against *Plasmodium falciparum* (Fiaz et al., 2013).

Conclusion

Plant based antimicrobials have enormous therapeutic potential as they can serve the purpose without any side effects that are often associated with synthetic antimicrobials. The extracts of the leaves and roots of *E. umbellata* showed excellent antimicrobial activity against the tested bacteria, fungus and yeast. Therefore, it is concluded that the extract of the leaves and roots of the plant can be regarded as good natural antibiotics with considerable degree of antimicrobial activity and that they can be used in the treatment of various infectious diseases caused by resistant microorganisms. The results also revealed that the use of roots of *E. umbellata* may be more beneficial than the aerial parts against infectious diseases. Further investigations are directed at isolation of pure compounds which are present in frac-

tions showing large inhibitory activity against various microorganisms as well as other pharmacological or toxicological properties aimed. Moreover, various parts of the plant may be used to treat various ailments as reported in the literature. Cultivation of this plant on commercial basis may also be employed so as to further increase the availability and to reduce the cost. Such usage may be more effective due to synergetic effect of various components rather than stand alone use of a pure compound.

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Optimization of oil extraction from high energetic potential plants performed through drying and solvent extraction methods

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The selection of species that accumulate oil with potential for biofuel production has favored advancements in the scientific and technological domains within the Brazilian biofuel program. The equipments and methods used for such selection have always prosecuted the objective of increasing oil extraction. The aim of this paper was to evaluate the yield of oil extracted from five vegetable species – castor oil plant (*Ricinus communis*), physic nut (*Jatropha curcas*), soybean (*Glycine max*), groundnut (*Arachis hypogaea L.*), and crambe (*Crambe abyssinica*) with two different moisture contents and using two different solvents, hexane and ethanol. The experimental design was factorial (5 × 2 × 2), in which grains of each of the five vegetable species in two different moisture contents were used for oil extraction. It can be concluded from this study that grain moisture content and solvent type had great influence in determining oil yield; and that hexane extraction was most efficient. *J. curcas* had the greatest increase of oil yield with 30.70% when performing extraction with grain moisture. The smallest differences between the solvents were found in the species *R. communis* with 12% of higher efficiency with hexane extraction.

Key words: Biodiesel, ethanol, hexane, oil extraction yield.

INTRODUCTION

The vegetable oil industry stands out within the agro-industry by the expressiveness of its economic stature. Vegetable oil is an important source of energy, essential fatty acids, vitamins and lipid-soluble antioxidants. The process for obtaining oleaginous grains and choosing the type of extraction process which they will go through depends on the initial oil content in the material. The methods must be simple and adaptable to various types of oleaginous (Singh and Bargale, 2000). Nowadays, the availability of oleaginous grains for supplying the agribusiness in the biofuels market has been increasing due to the need for compliance with the federal law that establishes the conditions for blending biodiesel into diesel. For that reason, it is important to plan and pursue solutions that will offer answers to issues that determine and influence the factors of production (Sartori et al., 2009). Different types of solvents, isopropanol, n-hexane, isohexane, acetone, methylpentanes, have been suggested in the literature (Wan et al., 1995; Apelblat et al., 1996; Batista et al., 1999; Kuk et al., 2005; Mohsen-Nia et al., 2007; Manic et al., 2011). Nowadays, there is a considerable interest in replacing these solvents (ethanol) by other alternative solvents, due to a growing concern regarding the environment and the safety of the process

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of oil production (Johnson and Lusas, 1983; Hammond et al.,2005). Among them, short-chain alcohols such as ethanol show the most promising results (Saxena et al., 2011).

The method that is mostly used for extracting oil from oilseeds is a petroleum distillate that contains a mixture of isomers of hexane (boiling points between 65 to 71°) industrially known as n-hexane. N-hexane can contain from 45 to 70% n-hexanes, as well as methylcyclopentane, 3-methylpentane, 2-methylpentane and cyclonhexane (Hammond et al., 2005; Wakelyn and Wan, 2006). Saxena et al. (2011) reported in a study, when comparing the yields of cottonseeds solvent extraction using hexane and ethanol at different temperatures, that higher efficiency was obtained using the renewable source ethanol as a solvent at higher temperatures. Brossard-González et al. (2010), when performing extraction with two different solvents (ethanol and hexane) and by pressing of physic nut seeds, concluded that extraction with ethanol as a solvent enabled higher yield when compared to pressing and no difference was found in the composition of the oil when using ethanol and hexane as solvents.

The higher moisture content in cold pressing reduces friction and results in low-yield and very low values of moisture effect the operation of the press (Singh and Bargale, 1990). Singh et al. (2002) showed that a decrease in moisture content and an increase in temperature improve oil extraction yield, thus reporting the importance of defining an optimum rage of moisture content. Solvent extraction is widely the most used method for oil extraction, which enables higher yield. However, there is little information about grain moisture content during the extraction process.

In the light of this consideration, this paper focuses on the energetic potential of oil extraction from energy crops. The aim of this study is to evaluate the extraction yield of oil from five species of plant using different solvents and different moisture contents.

MATERIALS AND METHODS

The experiment took place in a private laboratory in Cascavel-PR. The seeds used in the study were provided by Faculdade Assis Gurgacz (Assis Gurcacz College). The seeds were randomly chosen from different samples. Seeds of physic nut and castor oil plant were then shelled; all seeds were crushed in order to increase the surface area contacting the solvent. Seeds without moisture used in the tests were submitted to a drying process; moisture withdrawal was performed in an oven at 105°C to constant weight. Seeds with around 17% of moisture content were only crushed, weighted and then put into a soxhlet extractor. In this study, direct extraction process with hexane and ethanol solvents was performed in order to determine the oil content in the albumen. The soxhlet extraction method was used, based on procedures adapted from the analytical standards of the *Instituto Adolfo Lutz* (Adolfo Lutz Institute) (Pregnolato and Pregnolato, 1985), in order to determine the oil content of the seeds. Seeds were crushed in a portable mixer and then 5 g of the sample were weighted in filtering paper and transferred to the thimble of the soxhlet extractor. The flat bottom flask containing 200 ml of solvent was attached to the extractor. Heating was started to constant temperature and the extraction was carried out continuously for 8 h (four to five drops per second). The thimble was then removed. Solvents were distilled to a flask and the extracted residue remaining in the flask was dried in an oven at 105°C for 1 h and cooled in a desiccator to room temperature. The amount of extracted oil was then evaluated by the difference of the weights of the flat bottom flask with the oil and while empty.

The percentual (m/m) lipid or ethereal extract, E, was calculated according to the formula:

$$E = 100 \times N/P$$

Where N is the weight of lipids (g), and P is the weight of sample (g).

The experimental design was completely randomized factorial (5 × 2 × 2), with five vegetable species – castor oil plant (*Ricinus communis*), physic nut (*Jatropha curcas*), soybean (*Glycine max*), groundnut (*Arachis hypogaea L.*) and Crambe (*Crambe abyssinica*). Samples with two moisture contents were extracted with two solvents, hexane and ethanol, and four repetitions were done. The results were submitted to analysis of variance. The interaction between the factors, as well as their means were compared by Tukey's test at 1 to 5% error probability with the use of the statistics package Assistat® version 7.5 beta (Silva and Azevedo, 2002). Unfolding was performed when F was significant in the interaction.

RESULTS AND DISCUSSION

Table 1 shows the behavior of the analysis of variance, the significance of the treatments as well as the interacttion specie/moisture, specie/solvents and specie/moisture/solvent. The interaction between specie and solvent did not provide significant response for the oil yield of the studied species. It is possible to observe a significant effect on oil yield for the different vegetables ($P<0.01$) (Table 1). The species *J. acurcas* and *R. communis* excelled in oil income when compared to the others. Oil content of *G. max* was below the average established for the species studied in this paper. The seeds used in the experiment showed characteristics of oil content similar to the ones reported by Drummond et al. (2006), Kandpal and Madan (1995), Melhorança et al. (2010) and Rosseto et al. (2012). Table 2 shows the unfolding of the interaction of the studied factor (specie/moisture) for oil yield. It is possible to verify that the moisture content caused different responses for the studied genotypes. Extraction without moisture showed a significant increase in oil yield for all the analyzed species, emphasizing *R. communis*, which had an increase in oil content of 119%, compared to extraction with moisture. *J. acurcas* had the greatest increase of oil yield with 30.70% when performing extraction with moisture. No study similar to this one was found in the literatures.

Mpagalile et al. (2006) and Pighinelli et al. (2008) evaluated the effect of moisture content of *A. hypogaea* L. and *Helianthus annus* L., respectively, in oil extraction by mechanical pressing through which the second author found the range of 8 to 8.5% that would be the maximum

Table 1. Yield oil (%) of the species under different conditions.

Treatment	Oil content (%)
Specie	
G. max	8.96[d]
A. hypogaea	31.82[b]
J. curcas	37.07[a]
R. communis	37.32[a]
C. abyssinica	23.25[c]
Moisture content	
With 17% moisture	21.18[b]
Without moisture	34.19[a]
Solvent	
Hexane	34.36[a]
Ethanol	21.00[b]
CV (%)	10.86
Species (E)	**
Moisture (U)	**
Solvent (S)	**
E × U	**
E × S	**
U × S	n.s.
E × U × S	**

Coefficient of variation (CV %). Means with different small letters in the columns are statistically different at 1% (**) and 5% (*) probability; n.s, not significant. Tukey test.

Table 2. Unfolding of the interaction, for oil yield averages (%), according to specie/moisture and specie/solvent.

Specie	Moisture	
	With moisture	Without moisture
G. max	6.02[dB]	11.90[eA]
A. hypogaea	26.40[bB]	37.23[cA]
J. curcas	30.70[aB]	43.45[bA]
R. communis	23.41[bcB]	51.23[aA]
C. abyssinica	19.36[cB]	27.14[dA]

Specie	Solvent	
	Hexane	Ethanol
G. max	10.36[dA]	7.56[eA]
A. hypogaea	43.07[bA]	20.56[cB]
J. curcas	48.57[aA]	25.57[bB]
R. communis	39.39[bA]	35.25[aB]
C. abyssinica	30.43[cA]	16.07[dB]

oil yield. It is possible to conclude through the differences found in the unfolding of the interaction specie/solvent (Table 2) that the hexane solvent provided better oil yield. It is also notable the superiority of *J. curcas* in the extraction performed with hexane solvent, obtaining 48.57% of oil over ethanol extraction with 25.57%. The smallest differences between the solvents were found in *R. communis* with 12% of higher efficiency with hexane extraction. Melhorança et al. (2010) reported, when analyzing two solvents (hexane and methanol) for *J. curcas*, the

Table 3. Unfolding of interaction specie/moisture/solvent for oil yield (%).

Specie	Moisture/solvent			
	With moisture + hexane	With moisture + ethanol	Without moisture + hexane	Without moisture + ethanol
G. max	7.13^{cB}	4.91^{cB}	13.60^{dA}	10.20^{dAB}
A. hypogaea	40.75^{aA}	12.06^{bC}	45.40^{bA}	29.07^{bB}
J. curcas	43.70^{aB}	17.70^{abD}	53.45^{aA}	33.45^{bC}
R. communis	24.75^{bB}	22.07^{aB}	54.02^{aA}	48.44^{aA}
C. abyssinica	26.12^{bB}	12.60^{bD}	34.74^{cA}	19.54^{cC}

efficiency of hexane in the extraction; however, they emphasize that the cost-benefit is unfeasible because of its higher price. Brossard-González et al. (2010) found different values for *J. curcas* (31.22%) of average oil yield with hexane extraction compared to 34.34% with ethanol extraction. In this study, the authors reported the superiority of ethanol when compared to hexane solvent extraction and to pressing method extraction. Drumond et al. (2006), using ethanol as a solvent for extraction of seeds of *R. communis* obtained yields of 46.9% with ethanol, 51.1% with methanol and 41.1% with hexane. Kandpal and Madan (1995) obtained 37.4% inextraction of whole seeds and 46.0 to 48.6% with the albumen alone using petroleum ether solvent in the extraction of *J. cucas* in a soxhlet extractor. Oil yield was influenced by interaction specie/moisture/solvent (Table 3). Higher oil content was derived from combinations with no moisture and hexane solvent.

In this study, moisture content was a limiting factor. It is possible to observe that oil yield for *J. curcas*, even when extracted with ethanol that was the least efficient solvent in this study, was lower because of the moisture content in the extraction with hexane. *G. max*, when submitted to drying and hexane extraction, showed oil yield in conformity with the ones found in the literature. Ramesh et al. (1995) emphasize that drying or toasting processes optimize the extraction of oil from oilseeds and may affect the physical-chemical properties of the oil. Saxena et al. (2011) reported that the color of the oil extracted with ethanol solvent is a little bit darker than the color of oil extracted with hexane. Works that compare the extraction methods using biorenewable solvent and traditional fossil solvent (hexane) or pressing methods can be found in the literature by Brossard-González et al. (2010), Ribeiro et al. (2010), Ferreira-Dias et al. (2003) and Drumond et al. (2006). These authors emphasize that despite the better results of oil yield obtained by hexane extraction in some cases, its cost is high, and this can be a significant factor when compared to the low cost and the ease of use of ethanol; what adds to the much higher toxicity that hexane presents.

Conclusion

The grain moisture content and the solvent type used for oil extraction are two key factors that influence oil yield. The extraction with hexane solvent was significantly more efficient than the extraction with ethanol solvent, regardless of the moisture content. The species *J. curcas* and *R. communis* excelled in oil income when compared to the others.

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Full Length Research Paper

Application of glucose oxidase for the production of metal gluconates by fermentation

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The present study deals with the application of glucose oxidase (GOX) for the production of metal gluconates by fermentation method. It provides a method for the conversion of glucose into gluconic acid and its derivatives using the enzyme glucose oxidase (GOX). Due to the presence of calcium carbonate in fermentation medium the gluconic acid is converted into calcium gluconate. Conditions like concentration of substrate, temperature, pH, fermentation period and different phosphate sources were optimized during fermentation. The maximum GOX activity was observed at 35°C (pH 5.5) after 44 h of incubation at 100 rpm. At the maximum enzyme activity, the percentage yield of gluconates are also maximum; both go side by side. Sulphuric and oxalic acids method were employed for the production of gluconic acid. Derivatives of gluconic acid that is, calcium lactate gluconate, sodium gluconate, potassium gluconate, zinc gluconate and copper gluconate were formed by using double displacement and direct methods. The direct method gave the better yield. The percentage yields were 73, 89.63, 81.93, 92.86 and 81.53%, respectively.

Key words: Glucose oxidase (GOX), metal gluconate, double displacement.

INTRODUCTION

Gluconates are salts of gluconic acid. Gluconic acid is an organic compound with molecular formula $C_6H_{12}O_7$. In aqueous solution at faintly acidic pH, gluconic acid forms the gluconate ion. Gluconic acid, gluconate salts and gluconate esters transpire widely in nature because such compounds come up from the oxidation of glucose (Henk, 2006). The glucono- delta-lactone is a food item with valuable features like even pH development and impartial taste forming a favoured additive for diverse foods like bread, mozzarella, sea food, meat, tofu etc. (Znad et al., 2003). Also, it is transformed to gluconic acid by fermentation method or through electrophoresis (Ramachandran and Fontanille, 2006). By *Aspergillus nigar* in an air lift reactor, high calcium gluconates production is attained as pellet form of cell growth at modest specific growth rate and biomass concentration (Anastassiadiss, 2007). The gluconates in electrolyte is improved by cooling the mother liquor obtained after recovery of product is incessantly reused in additional batches (Dowdells et al., 2010).

Gluconic acid consists of a six-carbon chain with five hydroxyl groups and at the end is a carboxylic acid group. In aqueous solution, gluconic acid originates in equilibrium with the cyclic ester glucono delta lactone. Including its derivatives, calcium lactate gluconate is a blend of calcium lactate and calcium gluconate. In pharmaceutical, it is extensively used as pharmaceutical calcium basis in food and beverages; the outstanding characteristics with high solubility and natural taste go ahead for new application in an extensive range of

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finest product (Jungbunzlauer, 2001). Also, it shows the highest solubility among the calcium salts frequently used for mineral enhancement, with a preliminary solubility of 20 to 30% (Chicage, 1999).

Sodium gluconate is the sodium salt of gluconic acid formed by gulucose fermentation. Its aqueous solution is subject to oxidation or reduction even at high temperatures. However, biologically, it is simply degraded and thus having no waste water difficulty. Potassium gluconate is a loosely bound salt of potassium and gluconic acid. It had been used in technical applications for example, as confiscating agent in textiles or for galvanic surface treatment (Life Science Research Office, 1978). Zinc salt of gluconic acid is called zincgluconate. It is useful as it has lower microbial profile and a more whole reaction, yielding a product with a longer shelf life (Caruso, 2007). The present study relates to the "microbial production of metal gluconates". More specifically, this invention provides a method for the conversion of glucose into gluconic acid and its derivatives using the enzyme glucose oxidase (GOX).

MATERIALS AND METHODS

The conversion of glucose into gluconic acid and its derivatives can be followed by: analysis of the glucose content, analysis of the gluconic acid content, confirmation of gluconic acid, conversion of gluconic acid into gluconate, determination of the percentage yield of metal gluconate.

Micro-organism

The strain of *Aspergillus niger* was grown on potato dextrose agar (PDA) and malt extract agar medium at pH 5.5.

Slants preparation

PDA was prepared by dissolving 40.0 g of PDA in 1000 ml of distilled water. To make the clear solution, medium was first boiled with constant stirring up to 15 to 20 min and then poured in the cotton plugged sterilized test tubes.

Sterilization

The medium in the cotton plugged test tubes and flasks were sterilized in the autoclave at 121°C and 15 lbs/inch^2 for 20 min and the test tubes were placed in slanting positions for 24 h after calving.

Inoculation

The slants were inoculated with the fresh strain of *A. niger* with the help of inoculums needle and incubated in the incubator at 37°C for 24 h. After every two weeks, propagation of strain on the fresh medium was continued. The pure and identified colonies of *A. niger* were stored in cold incubator/refrigerator at 4°C.

Fermentation media

Submerged fermentation was used for the production of GOX from *A. niger* in 250 ml shake flasks. The composition of fermentation medium was described in previous research project (Shazia et al., 2011).

Optimization of conditions

For submerged fermentation, the conditions of substrate (carbon source), concentration of substrate, pH of media, temperature and fermentation period were optimized.

Substrate (carbon source)

Different type of carbon sources/substrates were wormed for submerged fermentation like glucose, fructose, sucrose and dextrin.

Concentration of substrate (glucose, sucrose)

The carbon source (glucose, sucrose) which was obligated for fermentation as a carbon source was 4.0, 5.0, 6.0, 7.0, 8.0, 9, 10, 11, 12 and 13%.

Media pH

The pH of the media was accustomed with 1 M HCl and 1M NaOH. The adjusted pH was 4.0, 4.5. 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0, these flasks were brewed in shaking incubator at 30°C and 100 rpm for 45 h.

Incubation time/fermentation period

Growth media filled with 10% glucose in 250 ml of flasks were incubated in shaking incubator at 100 rpm and 30°C. They were taken out for analysis after 12, 22, 44, 66 and 88 h of incubation.

Temperature effect

Different conditions of temperature were 25, 35, 45, 65 and 75°C.

Effect of different PO_4 source

The different phosphate group were KH_2PO_4 and K_2HPO_4.

Extraction of enzyme

The extraction was same as described in our previous research project (Shazia et al., 2011).

Assay method

The enzyme assay followed the method of Shazia et al. (2011). Enzyme activity was calculated by the following formula:

$$\text{Activity of enzyme at 25°C} = \frac{\Delta A \times V}{I \; A_{290} \; \mu}$$

Where ΔA = the absorbance of the sample, V = volume of reaction mixture (4 ml), I = length of the cell (2.0 cm), A_{290}mM = molecular

Table 1. Effect of substrate on GOX.

Substrate	Absorption	Enzyme activity (µ moles HQ/min/ml)
Glucose	0.098	1.69
Fructose	0.067	1.160
Dextrin	0.0421	0.72
Sucrose	0.0194	0.336

absorption co– efficient of hydroquinone at 290 nm, (2.31), µ = glucose oxidase solution, (0.05 ml).

By fixing the values, the aforementioned equation will become:

Enzyme activity = 17.316 × ∆A µmole HQ / min / mg / ml (Ciucu and Patroescu, 1984).

Estimation of calcium gluconate

The filtrate contained gluconic acid which is converted into Ca-gluconate in the presence of $CaCO_3$ in aforementioned fermentation medium. Its analysis in supernatant liquid was made by the method of pharmacopoeia 1990.

Required chemicals

2.0 ml of 3M HCl, 20 ml of 0.05M EDTA, 15 ml of 1M NaOH, and 300 mg hydroxynaphthol blue (indicator) were used for the study.

Methods

1 ml of sample solution, 2.0 ml of 3M HCl were diluted with distilled water upto 150 ml with steady stirring. About 20 ml of 0.05 M EDTA was added from burette. 15 ml of 1 M NaOH and 300 mg of hydroxy naphthol blue indicator was added up to royal blue end point. Each ml of 0.05 M EDTA is corresponding to 2.004 mg of Ca-gluconate. The %age yield of Ca-gluconate was determined by the following formula:

$$\%\text{age yield} = \frac{\text{Ca-gluconate produced}}{\text{Glucose added}} \times 100$$

Preparation of gluconic acid from calcium gluconate

Oxalic acid method

5 g Ca-gluconate was dissolved in 25 ml boiled water.1.5 crystallized oxalic acid was dissolved firstly in minimum amount of water. Both the solutions were mixed at 50°C with constant stirring; the content was filtered to remove calcium oxalate. Gluconic acid was crystallized at 30°C in oven.

Sulphuric acid method

Sulphuric acid was used in place of oxalic acid to remove calcium as calcium sulphate and released the gluconic acid. 5 mg Ca-gluconate was dissolved in 10 ml distilled boiled water. This solution was placed in ice bath then 15.5 ml 30 N sulphuric acid was added dropwise, the content was stirred constantly for about 5 min, and filtered to remove Ca-sulphate. Gluconic acid solution was obtained as filtrate.

Conformational test for gluconic acid

5 ml warmed aqueous solution of gluconic acid and 1 ml freshly prepared distilled phenylhydrazine was taken. The mixture was taken in a test tube and the content was heated in water bath minutes. Crystals of gluconic acid phenylhydrazide were formed and melting point was noted.

Preparation of gluconic acid derivatives or gluconates

The derivatives of gluconic acid were prepared either from Ca-gluconate or directly from the gluconic acid using the methods: double decomposition and direct methods.

Double decomposition method: Metal gluconate was prepared by the double decomposition of metal sulphate and Ca-gluconate. 8.0 mg Ca-gluconate was added in 20 ml boiled water, stirred to dissolve it and treated with 8.0 mg metal sulphate with constant heating and stirring. Ca-sulphate was precipitated which was removed by filtration. Related metal gluconate was concentrated at constant low temperature. Ethanol was added to crystallize the metal gluconate, which was dried and weighted to calculate the yield.

Direct method: Related metal gluconate was prepared by the direct method from metal carbonate and gluconic acid. Metal carbonate (5.0 mg) was dissolved in 50% solution of gluconic acid (78 g corresponding to 156 ml). The solution was heated to remove the CO_2. Sodium gluconate was concentrated under vacuum at constant heating at 30°C. The contents were crystallized, dried and weighted to calculate the yield.

RESULTS AND DISCUSSION

The present studies show that the microbial production of gluconates such as sodium, potassium, zinc, copper and Ca-lactate gluconate were prepared in their best yield by using direct method approach. The percentage yields of metal gluconates were maximum at the maximum enzyme activity. Both results are shown in comparison with each other (Tables 1 to 3).

Calculations

Factor = each ml of 0.05 M EDTA equivalent to 2.004 mg of Ca-gluconate.

Table 2. Effect of glucose concentration on GOX.

Concentration of glucose (%)	Absorption	Enzyme activity (µ,moles HQ/min/ml)
4	0.052	0.901
6	0.063	1.098
8	0.098	1.69
10	0.051	0.894
12	0.024	0.42
14	0.081	0.22

Table 3. Effect of glucose concentration on gluconate production by *A. niger.*

Concentration of glucose (%)	EDTA used	Calcium gluconate produced
4	18.51	37.1
6	26.64	53.4
8	32.68	65.5
10	24.05	58.2
12	22.75	45.6
14	19.36	38.8

Table 4. Effect of fermentation period on GOX from *A. niger.*

Fermentation period (h)	Absorption	Enzyme activity (µ moles HQ/min/ml)
2	0.091	1.58
22	0.147	2.31
44	0.138	2.54
66	0.099	1.82
88	0.08	1.39

Calculation for maximum reading

1 ml of 0.05 M EDTA was equivalent to 2.004 mg of Ca-gluconate; 32.68 ml of 0.05 M EDTA = (2.004). (32.68) = 65.50 mg. Our results are in accordance with the results of Mischak (1985) and Petruccioli and Federici (1993); they reported that 8% glucose concentration enhanced the reaction, while Ray and Banik (1999) reported that 15% glucose concentration was affective (Tables 4 and 5).

Calculation

Factor = each ml of 0.05M EDTA is equivalent to 2.004 mg of Ca-gluconate.

Calculation for maximum reading

1 ml of 0.05M EDTA is equivalent to 2.004 mg of Ca-gluconate. 20.30 ml of 0.05 M EDTA = (2.004). (20.30) = 40.68 mg. Our results are antagonistic because in our results, 44 h fermentation period is effective for enhanced production of enzyme and gluconates; while Fiedurck (1998) reported 72 h fermentation period is effective (Tables 6 and 7).

Calculation

Factor = each ml of 0.05M EDTA is equivalent to 2.004 mg of Ca-gluconate.

Calculation for maximum reading

1 ml of 0.05M EDTA is equivalent to 2.004 mg of Ca-gluconate. 19.73 ml of 0.05M EDTA = (2.004). (19.73) = 39.53 mg. Our results are in accordance with Wiebel and Bright (1971) and Iedruck and Grumeda (2000) (Tables 8 and 9).

Calculation

Factor = each ml of 0.05M EDTA is equivalent to 2.004

Table 5. Effect of fermentation period on Ca-gluconate production from *A. niger.*

Fermentation period (h)	EDTA used	Calcium gluconate produced
2	14.3	28.6
22	18.1	36.27
44	20.3	40.68
66	14.8	29.65
88	5.3	10.60

Table 6. Effect of pH on GOX production from *A. niger.*

pH	Absorption	Enzyme activity (µ moles HQ/min/ml)
4.0	0.054	0.936
4.5	0.080	1.391
5.0	0.098	1.432
5.5	0.087	1.692
6.0	0.058	1.01
6.5	0.025	0.44
7.0	0.011	0.199

Table 7. Effect of different pH for calcium gluconate production by *A. niger.*

pH	EDTA used (ml)	Calcium gluconate produced
4.0	16.1	32.26
4.5	18.8	37.71
5.0	17.2	34.48
5.5	19.7	39.53
6.0	15.7	31.52
6.5	14.0	28.07
7.0	13.3	26.65

Table 8. Effect of temperature on production of GOX by *A. niger.*

Temperature (°C)	Absorption	Enzyme activity (µ moles HQ/min/ml)
25	0.161	2.78
35	0.168	2.91
45	0.147	2.54
55	0.116	2.01
65	0.067	1.16

Table 9. Effect of temperature on Ca-gluconate production.

Temperature (°C)	EDTA used	Calcium gluconate produced
25	14.58	29.21
35	17.80	35.67
45	15.10	30.26
55	13.90	27.85
65	8.81	17.65

Table 10. Effect of different PO_4 sources.

Phosphate source	Glucose used (g/l)	EDTA used (ml)	Ca-gluconate produced	Percentage yield Ca-gluconate
KH_2PO_4	40	28.17	56.45	56.5
K_2HPO4	40	23.00	46.1	46.1

Table 11. Comparative percentage yield of metal gluconates.

Method	%yield Ca-lactate gluconate	% yield sodium gluconate	% yield pot-gluconate	% yield Zin-gluconate	% yield copper gluconate
Double decomposition method	68.04	87.35	78.97	81.01	80.00
Direct method	73.00	89.63	81.93	92.86	81.53

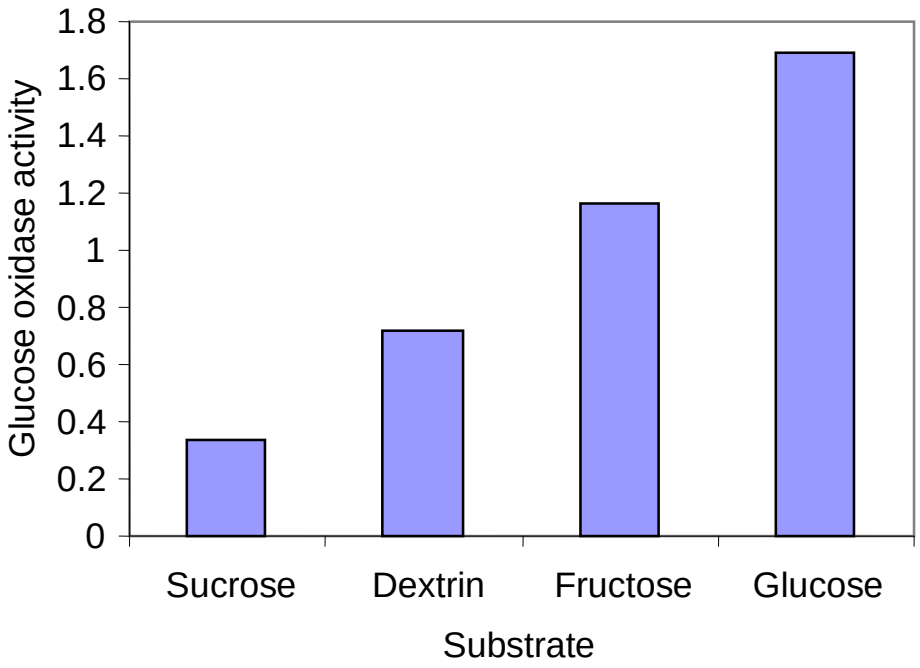

Figure 1. Effect of substrate on GOX.

mg of Ca-gluconate.

Calculation for maximum reading

1 ml of 0.05M EDTA is equivalent to 2.004 mg of Ca-gluconate. 17.80 ml of 0.05M EDTA = (2.004). (17.80) = 35.67 mg (Table 10).

Calculation

Factor = each ml of 0.05M EDTA is equivalent to 2.004 mg of Ca-gluconate.

Calculation for maximum reading

1 ml of 0.05M EDTA is equivalent to 2.004 mg of Ca-gluconate. 28.17 ml of 0.05M EDTA = (2.004). (28.17) = 56.45 mg. The results are in agreement with the work of Petruccioli and Federici (1993) (Table 11, Figures 1 to 10).

Conclusion

The present study shows that direct method gave better yield of metal gluconates as compared to double displacement method. These produced higher

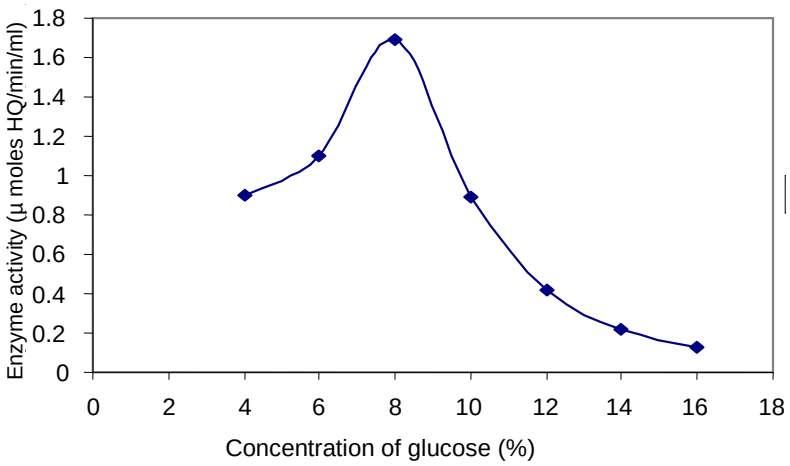

Figure 2. Effect of glucose concentration on GOX.

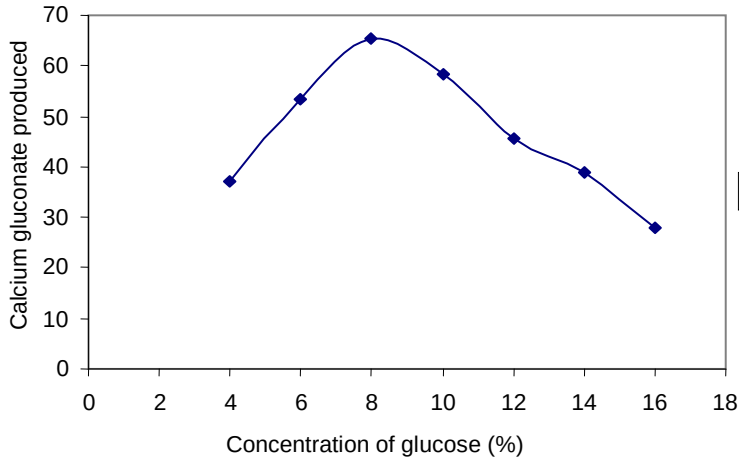

Figure 3. Effect of glucose concentration on gluconate production by *A. niger.*

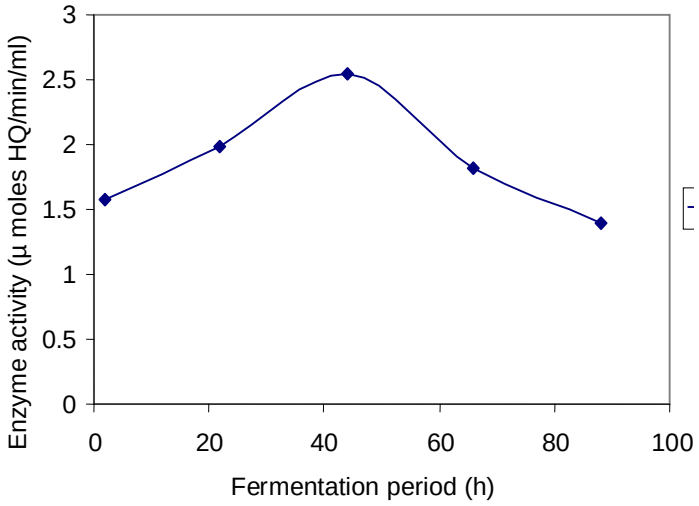

Figure 4. Effect of fermentation period on GOX from *A. niger.*

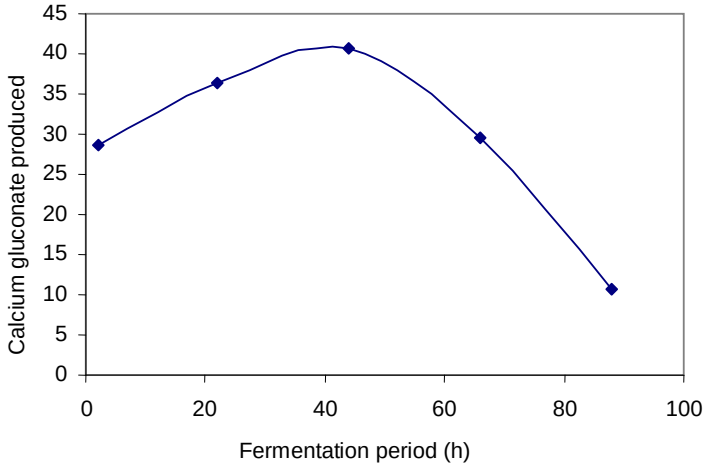

Figure 5. Effect of fermentation period on Ca-gluconate production from *A. niger.*

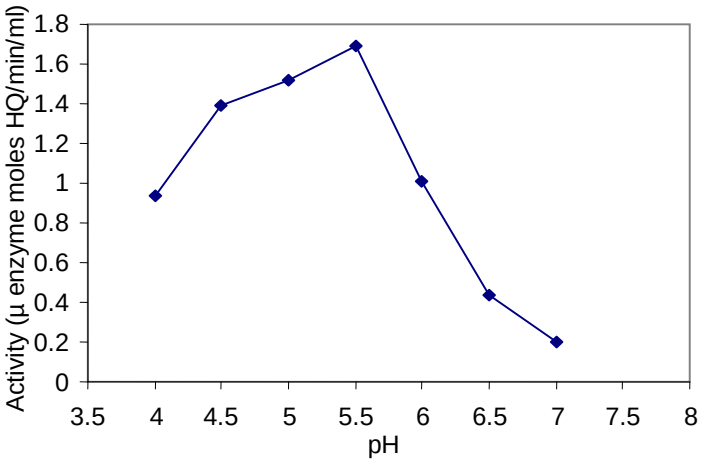

Figure 6. Effect of pH on GOX production from *A. niger.*

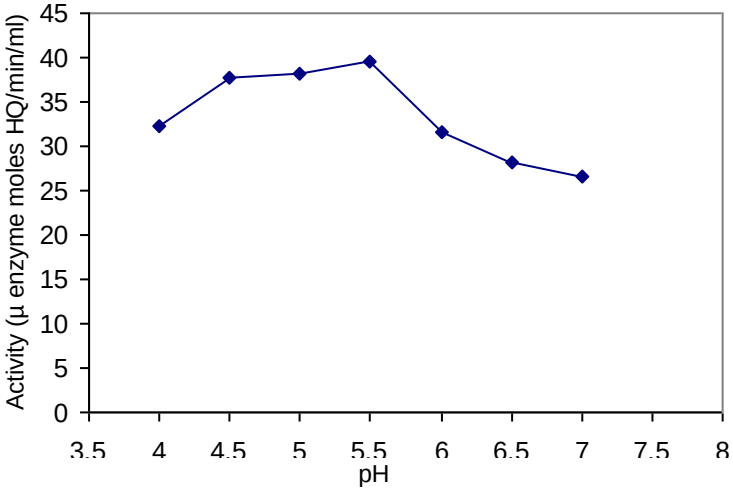

Figure 7. Effect of different pH for calcium gluconate production by *A. niger.*

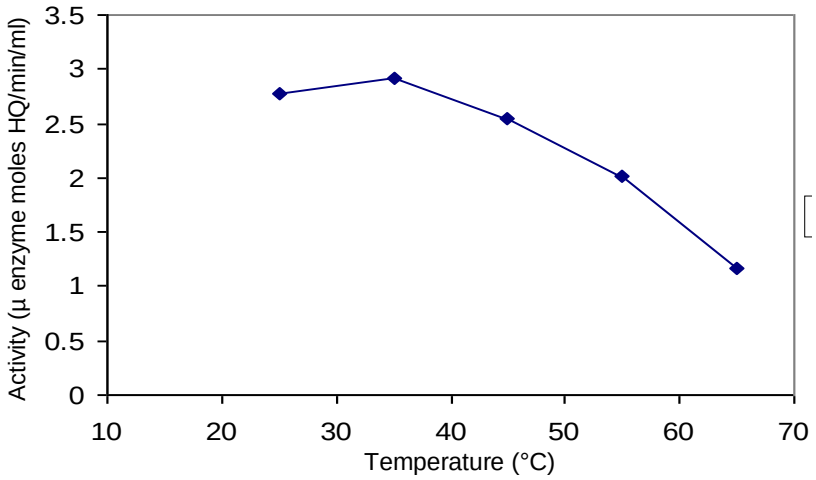

Figure 8. Effect of temperature on production of GOX by *A. niger.*

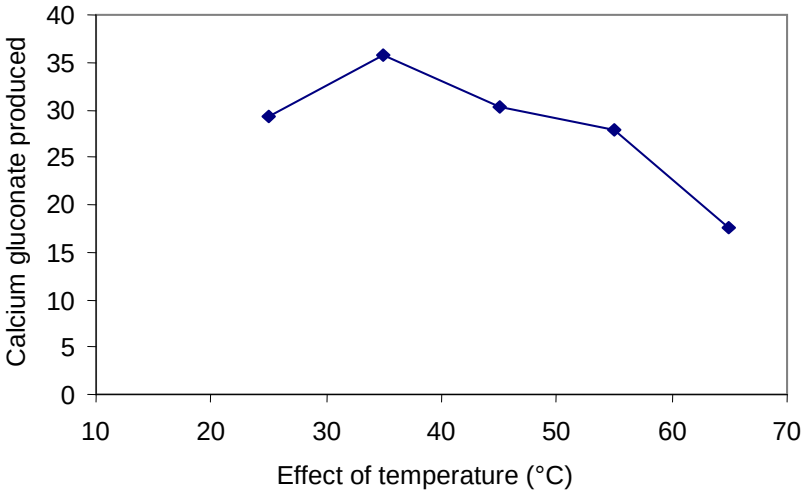

Figure 9. Effect of temperature on Ca-gluconate production.

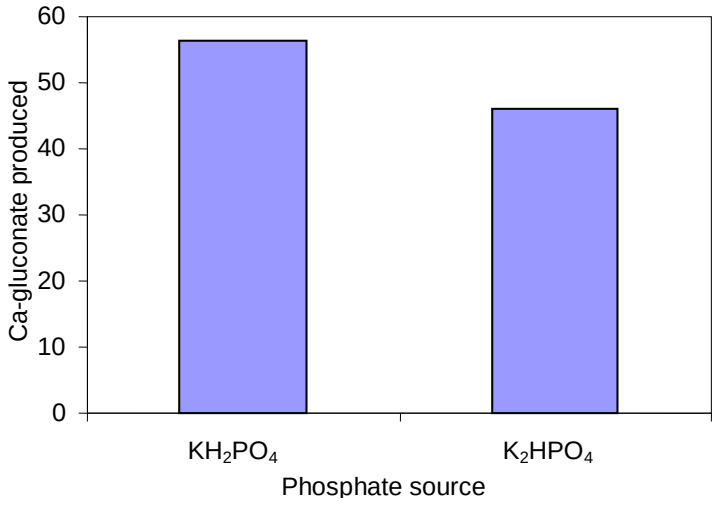

Figure 10. Effect of different PO_4 sources.

percentage yields at the maximum enzyme activity. The fermentation method is a cost effective method.

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Full Length Research Paper

Keratinase production by *Bacillus megaterium* RS1 using the statistical tool central composite design

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Keratinase producing bacterium *Bacillus megaterium* RS1 was obtained from feather dumping site of Rajapalayam. The optimal level of the key variables (starch, feather meal, calcium chloride) was used to determine the effect of their interactions on keratinase production using the statistical tool [(Central composite design (CCD) of response surface methodology (RSM)]. The second-order quadratic model with the optimum conditions [(starch (1%); feather meal (3%) and calcium chloride (0.02%)] was used. The nearness of the coefficient of determination (R^2 = 1.0000) to 1 ensures the satisfactory adjustment of the quadratic model to the experimental data. The maximum keratinase production was 142.9 U/ml.

Key words: Keratinase, Central composite design (CCD), response surface methodology (RSM), *Bacillus megaterium* RS1 starch, feather meal, calcium chloride.

INTRODUCTION

The incremental intensification in poultry industry all over the world resulted in the generation of millions of tonnes of chicken feather waste (Williams et al., 1990). Keratin a hard to degrade insoluble animal protein represents 90% of this keratinous waste (Bockle et al., 1995). This group of proteolytic enzymes which are able to hydrolyze insoluble keratins more efficiently than other proteases called keratinases and these belong to the extracellular enzymes (Rao et al., 1998). They are classified into various groups based on whether they are acidic, neutral or alkaline conditions. The protein chains are packed tightly either in α-helix (α-keratins) or in β-sheet (β-keratins) structures, which fold into a final 3-dimensional form (Kim, 2007). Microbial keratinase is an enzyme capable of degrading the insoluble structural protein found in feathers, hair, nail and wool. Keratin is a substrate by which sensitivity and resistance are closely linked to them fundamentally permitted of collagenase activity. High cysteine content is the most important property that differentiates keratins from other structural protein such as collagen and elastin (Sivakumar et al., 2012). These are poorly susceptible to digestion by enzymes such as trypsin, pepsin and papain (Gupta and Ramani, 2006). Keratinases from microbial source have many applications in feed, fertilizers, detergent, leather and pharmaceutical industries. Many organisms produce keratinase such as *Chrysoporium*, *Aspergillus*, *Alternaria*, *Trichurus*, *Curvularia*, *Cladosporium*, *Fusarium*, *Penicillium* (fungi), Streptomyces, *Vibrio*, *Mycobacterium* and *Bacillus* sp. (Vijay Kumar et al., 2011). Response Surface Methodology (RSM) is a statistical technique for the modelling and optimization of multiple variables, which determine optimum process conditions by combining experimental designs with interpolation by first or second polynomial equations in a sequential testing procedure (Ferreira et al., 2009). RSM has already been successfully applied for the optimization of enzymatic hydrolysis of other bioprocesses. Response surface methodology (RSM) is a useful tool which integrates mathematical and statistical approaches to analyze the effects of defined independent variables on the response without the need for prior knowledge of a predetermined

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Table 1. Independent variables and their coded levels for the central composite design used for keratinase production by *Bacillus megaterium* RS1.

Variable	-α	Low value	Coded variable	High value	+α
Starch	0.159104	0.5	1	1.5	1.8409
Feather meal	1.31821	2	3	4	4.68179
Calcium chloride	0.00318207	0.01	0.02	0.03	0.0368179

relationship between the response function and the variables. RSM is now considered as a standard statistical approach for designing experiments, building models, evaluating the effects of many factors and finding the optimal conditions for desirable responses and reducing the number of required experiments (Coninck et al., 2000). Response surface methodology was used to optimize the conditions for the extracellular production of keratinase (Siva et al., 2012a).

Optimization of the fermentation process parameters through a statistical approach, such as 'central composite design' and response surface methodology (RSM), has been well appreciated for a significant improvement in yield as well as a decrease in the production cost of the enzyme (Sivakumar et al., 2012b). Therefore, this study was mainly focused on statistical optimization of keratinase production using central composite design for high yield with low cost. In this work, RSM was adopted to determine the optimal conditions for the production of keratinase from *Bacillus megaterium* RS1 and the interacttions among the factors that influence the response of the keratinase production were determined.

MATERIALS AND METHODS

Optimization of significant variables for keratinase production using CCD

To find the optimal cultivation conditions for keratinase production, CCD with five coded levels was used for locating the true optimum conditions of starch (carbon source), feather meal (substrate concentration) and calcium chloride (metal ions). For the three factors, this trial was essentially a full 2^3 factorial design with six axial points (α = 1.68) and six replication of the centre points, resulting in a total number of 20 experiments. The levels of the variables and the experimental design are shown in Table 1. The results of CCD were expressed as the following second-order polynomial (Equation 2) using a multiple regression technique.

$$Y = \beta_o + \Sigma \beta i \chi i + \Sigma \beta ii \chi i^2 + \Sigma \beta ij \chi i \chi j$$

Where, Y is the predicted response, β0 the intercept term, βi the linear coefficients, βii the quadratic coefficients, βij the interactive coefficients, xi and xj the coded independent variables (Song et al., 2007).

Keratinase production by optimized parameters

After 48 h of incubation on optimized medium [starch (carbon source), 1% yeast extract (nitrogen sources); 0.5%, calcium chloride (metal ions); 0.2%, PEG (surfactants); 0.02%, inoculum concentration (2%), 3% feather meal (substrate concentration) at pH 7.0, 40°C) the culture medium was centrifuged at 5000 rpm for 15 min. The supernatant was used as crude enzyme source for keratinase assay. Keratinase activity was assayed as per the method of Burtt and Lchida (1999) using Azocasein. About 5 mg of Azocasein was added to a 1.5 ml centrifuge tube along with 0.8 ml of 50 mM potassium phosphate buffer (pH- 7.5) at 37°C for 1 h with constant agitation (900 rpm). This mixture was agitated until the Azocasein was completely suspended. Then, 0.2 ml aliquot of supernatant (crude enzyme) was added to the Azocasein, mixed and incubated for 15 min at 50°C with shaking. The reaction was terminated by adding 0.2 ml of 10% of trichloroacetic acid (TCA). The reaction mixture was filtered and analyzed for activity. The absorbance of the filtrate was measured at 450 nm with a UV-160 spectrophotometer. A control sample was prepared by adding TCA to a reaction mixture before the addition of enzyme solution. The unit of keratinase activity was measured at 0.01 unit increase in the absorbance at 450 nm as compared to the control after 15 min of reaction. Standard curve was performed with tyrosine and the enzyme activity was expressed in units.

Statistical analysis

Experimental designs and the polynomial coefficients were calculated and analyzed using a trial version of Design-Expert software (version 8.0.4, Stat-Ease Inc., Minneapolis, USA). Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

RESULTS

Central composite design (CCD) and response surface methodology (RSM)

The optimal level of the key variables (starch, feather meal and calcium chloride) and the effect of their interacttions on keratinase production were further explored using the CCD of RSM. The design matrix and the corresponding experimental data to determine the effects of three independent variables are shown in Table 1. The mutual interactions between every two of the three variables which were significant under the optimum condition, the predicted maximum keratinase production were calculated as 142.9 U/ml. By applying multiple regression analysis to the experimental data (Table 2), the following second order polynomial equation was established:

Terms of coded factors

$$\text{Keratinase} = +142.89 - 7.11*A + 8.78*B - 6.11*C - 2.94*A*B - 0.34*A*C - 1.64*B*C - 27.01*A^2 - 0.16*B^2 - 13.68*C^2$$

Table 2. Variance analysis of response surface quadratic model for keratinase production by *Bacillus megaterium* RS1.

Source	Sum ofsquares	Df	Meansquare	F-value	p-value prob > F
Model	24797.15	9	2755.24	81468.99	< 0.0001[c]
A-starch	690.31	1	690.31	20411.50	< 0.0001[c]
B-feather meal	1052.89	1	1052.89	31132.53	< 0.0001[c]
C-calcium chloride	596.58	1	596.58	17640.15	< 0.0001[c]
AB	69.03	1	69.03	2041.17	< 0.0001[c]
AC	0.91	1	0.91	26.94	< 0.0001[c]
BC	21.45	1	21.45	634.29	< 0.0001[c]
A^2	10304.33	1	10304.33	3.047E+005	< 0.0001[c]
B^2	12850.99	1	12850.99	3.800E+005	< 0.0001[c]
C^2	4851.80	1	4851.80	1.435E+005	< 0.0001[c]
Residual	0.34	10	0.34		
Lack of fit	0.34	5	0.068		
Pure error	0.000	5	0.000		
Cor total	24797.49	19			

R^2 = 1.0000; Adj R^2 = 1.0000; CV% = 0.20; [c]Model terms are significant.

Terms of actual factors

Keratinase = 320.02917 + 220.81347* Starch + 198.90234* feather meal + 5420.42848* calcium chloride - 5.87500* starch* feather meal 67.50000* starch* calcium chloride 163.75000* feather meal* calcium chloride - 108.03198* starch^2 - 30.16134* feather meal^2 - 1.36808e + 005* calcium chloride^2 2

Where, Y1 was the keratinase production, X1 the starch, X2 the feather meal and X3 the calcium chloride.

The model F-value of 81468.99 implies the model is significant. The "model F-value" is 0.01% occurence due to blare. Values of "Prob > F" less than 0.0500 indicate that the model terms are significant. In this case A, B, C, AB, AC, BC, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "pred R-squared" of 0.9999 is in reasonable agreement with the "adj R-squared" of 1.0000. "Adeq precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Our ratio of 768.125 indicates an adequate signal. This model can be used to navigate the design space.

In the present study, all these results showed a good agreement between the experimental and predicted values and implied that the mathematical models were suitable for the simulation of keratinase production (Tables 3 and 4).

DISCUSSION

The enlightening of microbial keratinase production is the aim of these investigations, being the production capacity of the organism depending on the successful selection of growth conditions and substrate. The strain *B. megaterium* RS1 showed higher keratinase production on mutual interactions between every two of the three variables which is an inexpensive and readily available substrate. Thus, the utilization of such substrate may result in a cost effective process. The three-dimensional response surfaces (Figure 1a, b, c) and contour plots are shown in Figure 2a, b, c (keratinase production) which depicts the interactions between the two variables by keeping the other variables at their zero levels. The shapes of the contour plots, circular or elliptical, indicate whether the mutual interactions between the variables are significant or not. A circular contour plot of response surfaces indicates that the interaction between the cor-responding variables can be ignored, while an elliptical or saddle nature of the contour plot suggests that the inte-raction between the corresponding variables is significant (Shankar and Isaiarasu, 2012). The second-order quadratic model with the optimum conditions (starch - 1%; feather meal- 3% and calcium chloride - 0.02%) resulted in a maximum titre of 142.9 U/ml of keratinase at 48 h. The nearness of the coefficient of determination (R^2 = 1.0000) to 1 ensures the satisfactory adjustment of the quadratic model to the experimental data. Likewise, model of RSM was employed in the optimization of major keratinase producing conditions such as starch, feather meal and calcium chloride.

Optimization has one of the most important criteria when it comes to developing any new microbial process. The box-Brinker design experiment is used to determine the maximum keratinase production at the most adequate pH, temperature and catalyst concentration (Anbu et al., 2005). Response surface methodology, an experiment strategy for seeking the optimum conditions for a multi-variable system, is a much more efficient technique for optimization. This method has been successfully applied

Table 3. Central composite design for keratinase production by *Bacillus megaterium* RS1.

Standard	Run	Factor 1	Factor 2	Factor 3	Keratinase (U/ml)
		Starch (%)	Feather meal (%)	$CaCl_2$ (%)	
15	1	1.00	3.00	0.02	142.9
3	2	0.50	4.00	0.01	98.1
12	3	1.00	4.68	0.02	72.7
20	4	1.00	3.00	0.02	142.9
8	5	1.50	4.00	0.03	62.5
1	6	0.50	2.00	0.01	71.5
10	7	1.84	3.00	0.02	54.9
9	8	0.16	3.00	0.02	78.8
2	9	1.50	2.00	0.01	63.7
7	10	0.50	4.00	0.03	83.4
5	11	0.50	2.00	0.03	63.1
6	12	1.50	2.00	0.03	54.2
18	13	1.00	3.00	0.02	142.9
4	14	1.50	4.00	0.01	78.8
17	15	1.00	3.00	0.02	142.9
19	16	1.00	3.00	0.02	142.9
11	17	1.00	1.32	0.02	43.2
14	18	1.00	3.00	0.04	76.1
13	19	1.00	3.00	0.00	100.5
16	20	1.00	3.00	0.02	142.9

Table 4. The matrix of the CCD experiment and the corresponding experimental data by *Bacillus megaterium RS1.*

Standard	Run	Factor 1	Factor 2	Factor 3	Actual value	Predicted value
		Starch (%)	Feather meal (%)	Calcium chloride (%)		
15	1	1.00	3.00	0.02	142.9	142.8
3	2	0.50	4.00	0.01	98.1	98.2
12	3	1.00	4.68	0.02	72.7	72.5
20	4	1.00	3.00	0.02	142.9	142.8
8	5	1.50	4.00	0.03	62.5	62.6
1	6	0.50	2.00	0.01	71.5	71.5
10	7	1.84	3.00	0.02	54.9	54.7
9	8	0.16	3.00	0.02	78.8	78.6
2	9	1.50	2.00	0.01	63.7	63.8
7	10	0.50	4.00	0.03	83.4	83.4
5	11	0.50	2.00	0.03	63.1	63.3
6	12	1.50	2.00	0.03	54.2	54.2
18	13	1.00	3.00	0.02	142.9	142.8
4	14	1.50	4.00	0.01	78.8	78.8
17	15	1.00	3.00	0.02	142.9	142.8
19	16	1.00	3.00	0.02	142.9	142.8
11	17	1.00	1.32	0.02	43.2	43.0
14	18	1.00	3.00	0.04	76.1	75.9
13	19	1.00	3.00	0.00	100.5	100.3
16	20	1.00	3.00	0.02	142.9	142.8

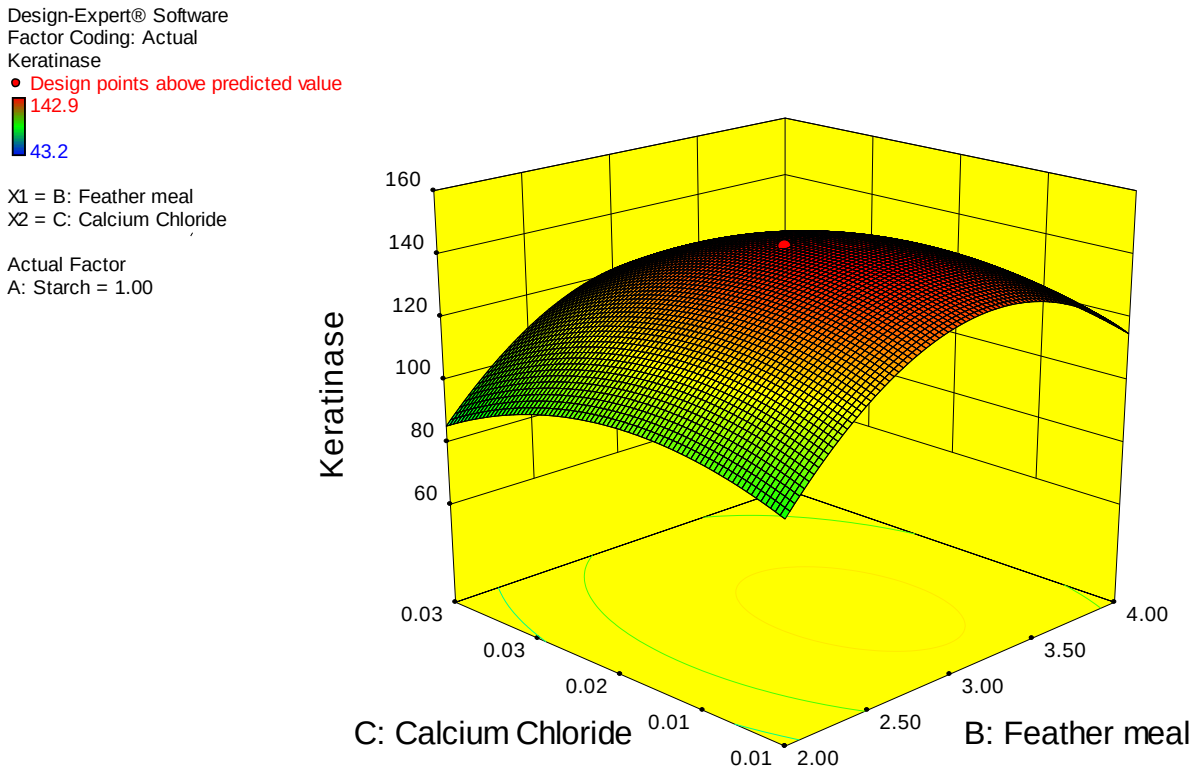

Figure 1a. Interaction between calcium chloride and feather meal.

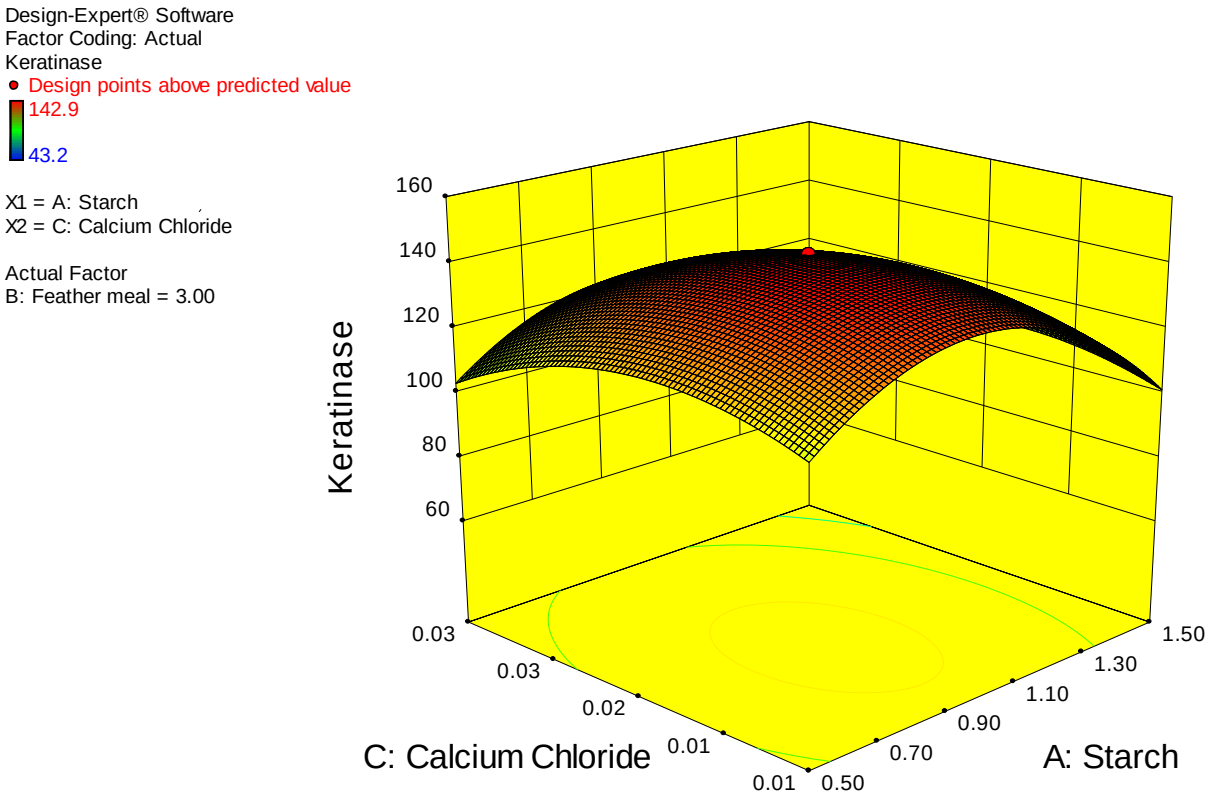

Figure 1b. Interaction between calcium chloride and starch.

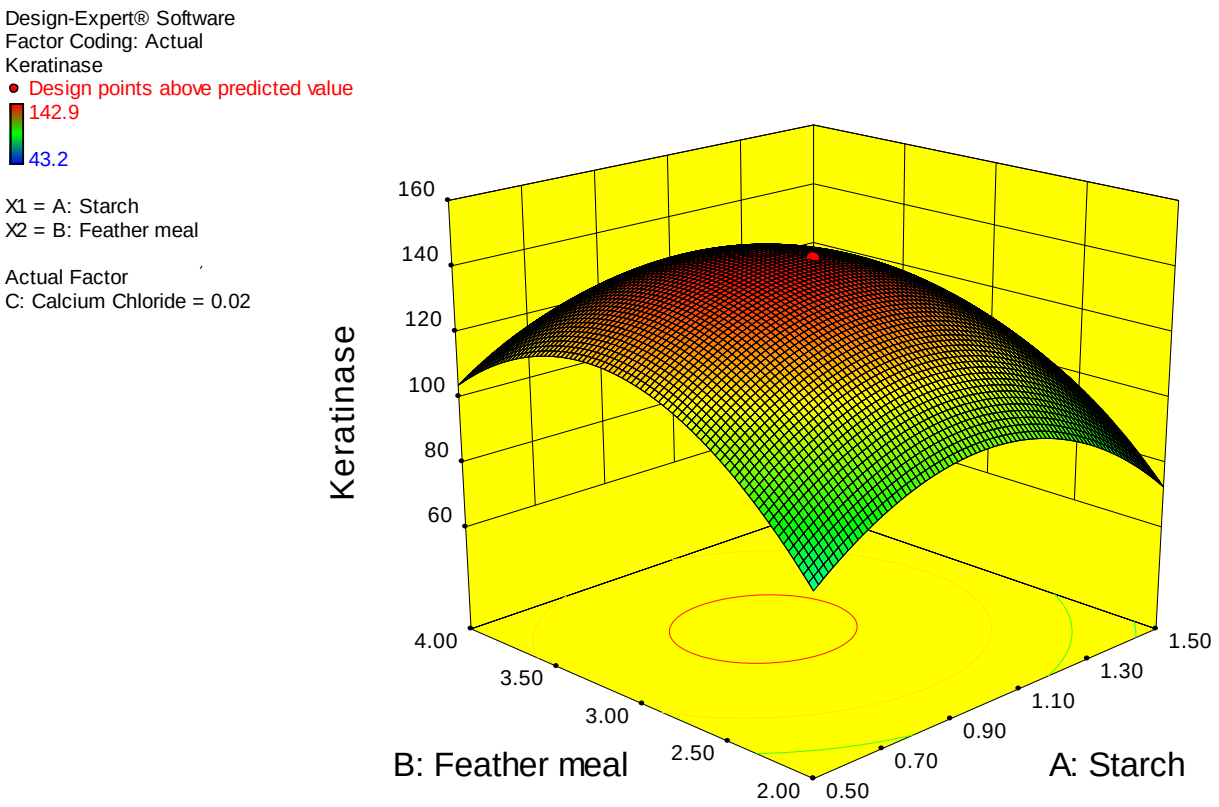

Figure 1c. Interaction between feather meal and starch.

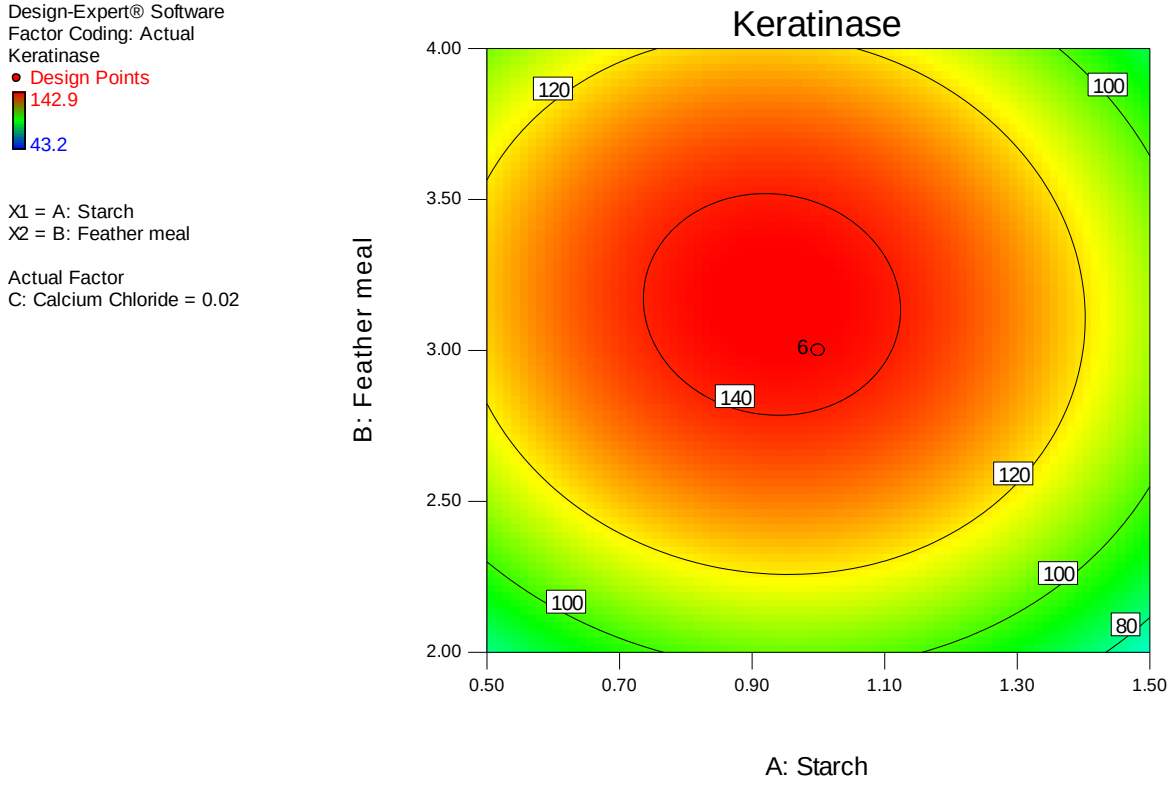

Figure 2a. Contour plot for feather meal and starch.

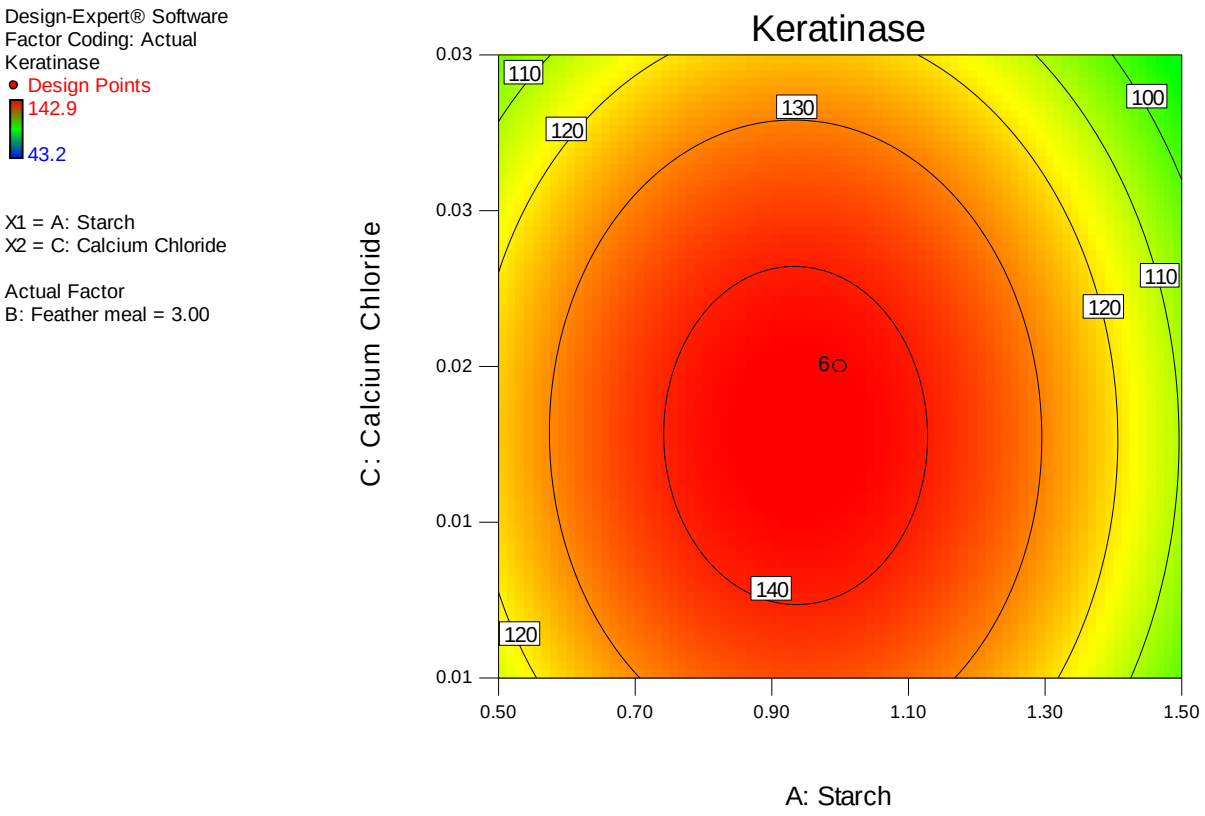

Figure 2b. Contour plot for calcium chloride and starch.

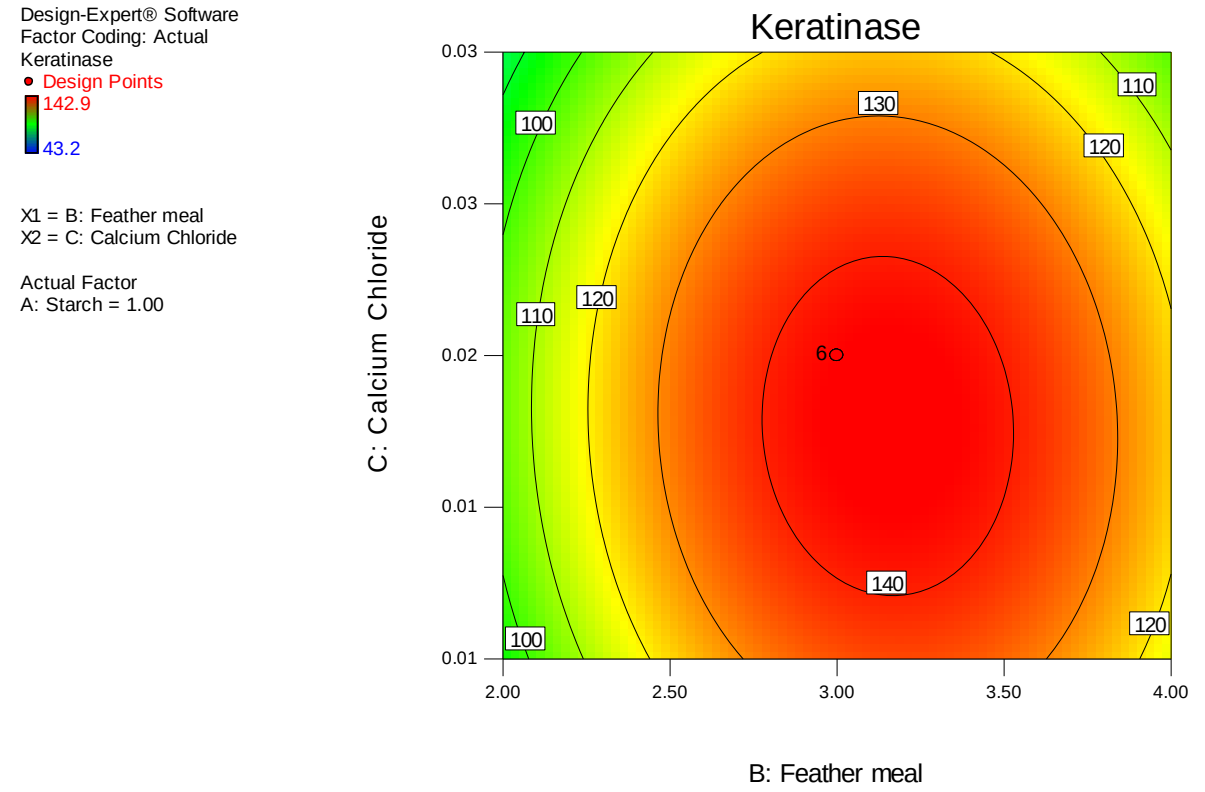

Figure 2c. Contour plot for calcium chloride and feather meal.

for media optimization in different fermentation process as well as for establishing the conditions of enzymatic hydrolysis and sulfuric acid production. To develop a process for maximum production of keratinase from poultry feather, standardization of media components is crucial. Isolated species of *Streptomyces* sp. is capable of rapidly degrading native feather. The production of keratinase was achieved by *Streptomyces* sp. using CCD and RSM (Tatineni et al., 2008). A two-step RSM study is conducted for the optimization of keratinase production and enzyme activity from poultry feather by *Streptomyces* sp 7. Initially, different combinations of salts are screened for maximal production of keratinase at a constant pH of 6.5 and feather meal concentration of 5 g/l. A combination of K_2HPO_4, KH_2PO_4 and $NaCl_2$ gives a maximum yield of keratinase (70.9 U/ml) production. In the first step of the RSM study, the selected five variables (feather meal, K_2HPO_4, KH_2PO_4, $NaCl_2$ and pH) are optimized by a 2^5 full-factorial rotatable central composite design (CCD) that has resulted in 95 U/ml of keratinase production. The results of analysis of variance and regression of a second-order model show that the linear effects of feather meal concentration ($p < 0.005$) and $NaCl_2$ ($p < 4.731$ e-6), KH_2PO_4 ($p < 1.01$e-10) and pH (p 7.63e-7) are more significant than the linear and interactive effects of the process variables. These optima are pH 11.0, temperature of 45°C at 300 rpm (Radhika et al., 2007).

The RSM applied to the optimization of keratinase production in the investigation suggested that the importance of verity of factors at different levels, the central composite design (CCD) exploited in the present study enabled as to study and explore the culture conditions, which would support a 3.4 fold increase in keratinase production. The high degree of similarity was observed between the predicted and experimental values that reflected the accuracy and applicability of RSM to optimize the process for enzyme production. RSM was successfully applied to the production of keratinase by Zauari et al. (2010). On the other hand, *Bacillus pumilus* AI showed the maximum production was 87.73 U/ml. Likewise, Sivakumar et al. (2012c) stated that the maximum keratinase enzyme production was 63.01 and 60.67 U/ml obtained by *Bacillus cereus* TS1 and *Bacillus thuriengiensis* TS2, respectively. There were three factors namely pH, temperature and starch used for RSM optimization in *B. cereus* TS1 and pH, temperature, mannitol were used for RSM optimization in *B. thuriengiensis* TS2. In this context, the present study similar to that of Matsui et al. (2009) having large research aimed to isolate feather degrading microorganism and investigated the characterization of feather degrading enzyme for socioeconomic importance.

Conclusion

The present work of the optimum cultural conditions for keratinase production by *B. megaterium* RS1 species was studied by RSM using central composite design with three variables (starch, feather meal and calcium chloride) for maximizing the production of keratinase.

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African Journal of Biotechnology

Full Length Research Paper

Seasonal variation of plasma testosterone levels in Algerian male Arabia goats

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The aim of the present work was to evaluate the general monthly averages of the testosterone hormone and the influence of season and photoperiod on plasma testosterone levels in Arabia bucks of Algeria. Testosterone concentrations were determined throughout one year in blood samples collected every fortnight of the month from nine males of Arabia goat breeds, fed a constant diet, maintained without interactions with female goat. Plasma testosterone level was measured by radioimmunoassay. Analyses performed show that the monthly averages of the testosterone hormone vary during the year; higher levels were recorded during August (T=8.57±6.72, P=0.00*) however, bucks displayed the same change tendency during the same period (P=0.79). Plasma testosterone concentrations vary significantly between seasons (P=0.00***) being higher during autumn (6.15±3.81ng/ml) compared with spring (0.90 ± 1.27ng/ml) when hormone synthesis reaches its lowest levels. In conclusion, Arabia bucks of Algeria displayed a clear seasonality of plasma testosterone concentration with very low levels in winter and spring (from January to May) and high levels in summer and autumn (from July to November).**

Key words: Arabia bucks, testosterone, season, photoperiod.

INTRODUCTION

The balance of the mechanisms that controls reproduction is based on the permanent relation between the central nervous system and the gonads, a relation which is ensured by the gonadotrophic and the steroidian hormones. However, this control is modified by several external factors (Chemineau and Delgadillo, 1994). Photoperiodic changes related to season have a direct effect on the reproductive activities due to a change in the levels of the hormones secreted by hypothalamus, pituitary, epiphysis and gonads (Pérez and Mateos, 1995).

At intermediate and higher latitudes, the breeding season begins when the duration of daylight becomes shorter (autumn) and ends in winter, when the photoperiod is increasing. However, considerable variation exists between goat breeds (Restall, 1992; Mascarenhas et al., 1995; Zarazaga et al., 2005). Male goats originating from the temperate regions generally display marked seasonal variation in reproductive activity. The decrease in day length in autumn is accompanied by an increase in gonadotrophic hormone production and testosterone levels, with maximum secretion (Delgadillo and Chemineau, 1992).

Testosterone is the hormone responsible for spermatogenesis and sexual behaviour, thus the seasonal pattern of testosterone secretion could limit the male reproductive efficiency during some periods of the year (Chemineau and Delgadillo, 1994). The study carried by Hammoudi et al. (2010) on two parameters of the sexual activity of Arabia bucks (scrotal circumference and sexual

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behaviour) shows that the intensity of sexual activity varies according to the season of the year with a maximal activity during summer and autumn. The aim of this research is to determine the changes in the levels of the testosterone hormone in the blood serum of male Arabia goats. Thus, this will be a possible contribution to this field of research on Arabia goats where limited numbers of researches are available and these results may be useful in the determination of the reproduction season.

MATERIALS AND METHODS

Location and animals

The study was conducted on an experimental farm of the University of Tiaret in western Algeria (latitude of 35°15' N and longitude of 1°26' E). This region is situated in the high plateau of Algeria, a semi-arid area characterized by cold and humid winter and hot and dry summer. Temperatures vary between 0.7 and 12.7°C in winter and between 13.5 and 36.2°C in summer. The annual rainfall is 389.3 mm for the year of experiment. The daily photoperiod varies between 9 h 37 min during the solstice of winter and 14 h 23 min during the solstice of summer. Nine sexually mature Arabia bucks aged 4 were used in the study. During the trial, the animals were maintained permanently under natural photoperiod and housed separately from the female goat. All bucks received a daily diet of 500 g barley, and had free access to straw, mineral additive block and freshwater.

Blood sampling and hormonal assay

In this study, for the annual fluctuation of testosterone levels, a single blood samples were collected every fortnight from all male goats throughout the year (January 20011-december2011). A total of 216 samples were analyzed. Blood sample collection began 2 h after the expected dawn time (National Meteorological Institute, Meteorological Calendar 2011). Sampling was done via the jugular vein into heparinized 4 ml tubes, and immediately placed in ice box. The samples were then transported to the laboratory and centrifuged at 3000 round per min during 5 min. The serum was recovered and stored at −20°C in duplicate, until analyzed for plasma testosterone concentration, using a commercial RIA kit (Immunotech A Beckman Coulter Company: RIA Testosterone, direct REF: 1119). The minimum detectable plasma testosterone concentration for the assay was 0.1 ng/ml. The hormone analyses were performed at the pharmaceutical laboratory of the nuclear center research of Draria in Algiers.

Statistical analysis

In order to determine any possible significant effects of the months, seasons and photoperiod on the testosterone variations for the Arabia bucks, one way variance analysis was performed using the procedure GLM using the software SPSS package (Chicago,1986). Multiple comparisons of means were analyzed using the Student-Newmen-Keuls test. The factors used in the statistical model (months, seasons and photoperiod) are not random factors; they depend on the geographical position or the latitude of the study region.

RESULTS

Annual evolution of testosterone

Our results show that the testosterone levels start to increase sensitively from June to reach a maximal value during the month of August (8.57 ± 6.72 ng/ml), then they start to fluctuate between September and November and decrease remarkably in December reaching minimal values in May (0.52 ± 0.54 ng/ml) (Figure 1). Despite displaying the same changes tendency of testosterone hormone levels during the same period (P=0.79), bucks individual reproductive performances seem to be variable.

Testosterone seasonal variations

Testosterone levels follow seasonal variations during the year. Maximal values were recorded in autumn (6.15 ± 3.81 ng/ml) then they begin to decrease progressively during winter (1.06 ± 1.42 ng/ml) reaching the minimal averages in spring (0.90 ± 1.27 ng/ml). From summer, the testosterone seasonal mean value increases again (4.81 ± 5.30 ng/ml) (Figure 2).

For the male Arabia goat, the difference between the testosterone seasonal average in summer and autumn was statistically significant from that in winter and spring (P=0.00). But, the difference was not significant between the testosterone seasonal average in summer - autumn and in winter - spring (P=0.05, P=0.82, respectively). On the other hand, the smelling of bucks varies around the year being more pronounced in summer and autumn.

Comparison between photoperiod and testosterone levels

Results show that the increase in the testosterone levels coincides with the decrease of the day-length (summer solstice) and a remarkable decrease in the testosterone levels coincides with the increase in the day-length (winter solstice) (Figure 3).

DISCUSSION

The present study demonstrated that Arabia bucks maintained under a natural photoperiod show marked seasonal variation of testosterone secretion. These variations were recorded despite feeding animals with a constant diet. The results indicate that season strongly influences testosterone secretion of these animals. Hammoudi et al. (2010) demonstrated that in male Arabia goats, both exual behavior and scrotal circumference follow seasonal variations during the year. They attain maximal values in autumn (7.96 ± 1.28 and 26.89 ± 0.55 cm, respectively) then they decrease progressively during winter (6.09 ± 1.25 and 25.65 ± 0.27 cm, respectively) to reach the minimal averages in spring (4.89 ± 1.66 and 25.41 ± 0.37 cm). In summer the values start again to increase (7.70 ± 0.67 and 27.43 ± 0.40 cm).

The testicular activity of bedouine breed bucks of in the south-west of Algeria shows clear seasonal variations with a maximum in summer and autumn (July-August-

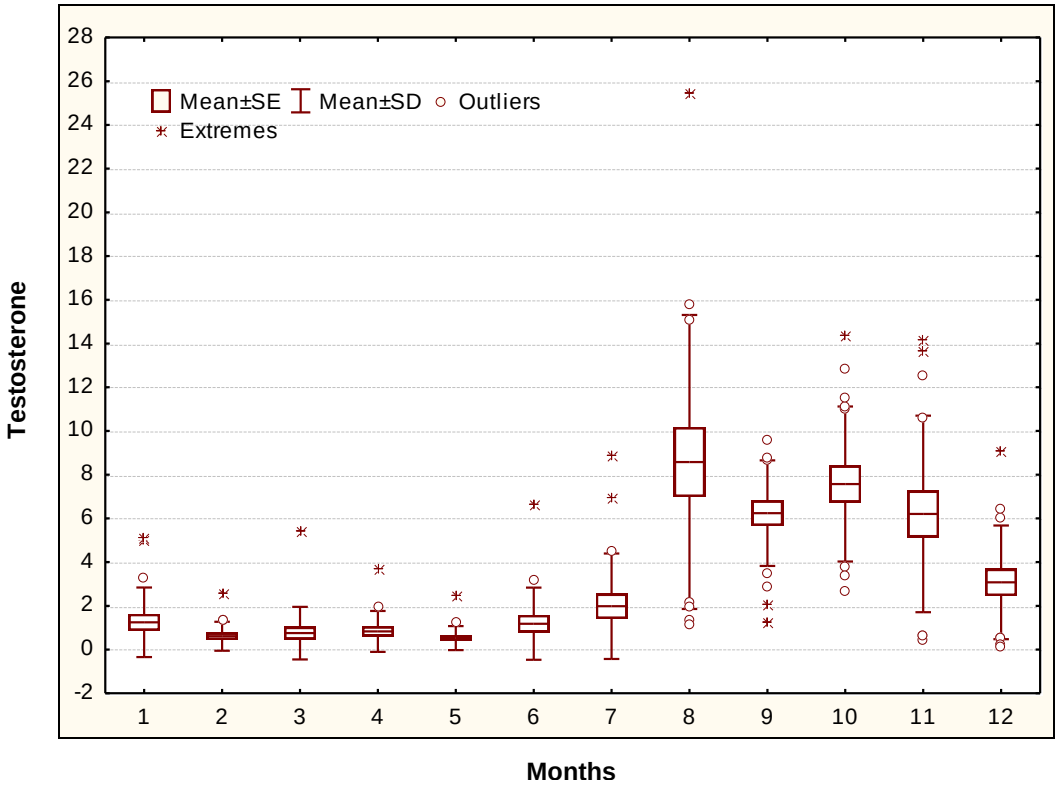

Figure 1. Testosterone monthly mean levels of male Arbia goat. 1, January; 2, February; 3, March; 4, April; 5, May; 6, June; 7, July; 8, August; 9, September; 10, October; 11, November; 12, December.

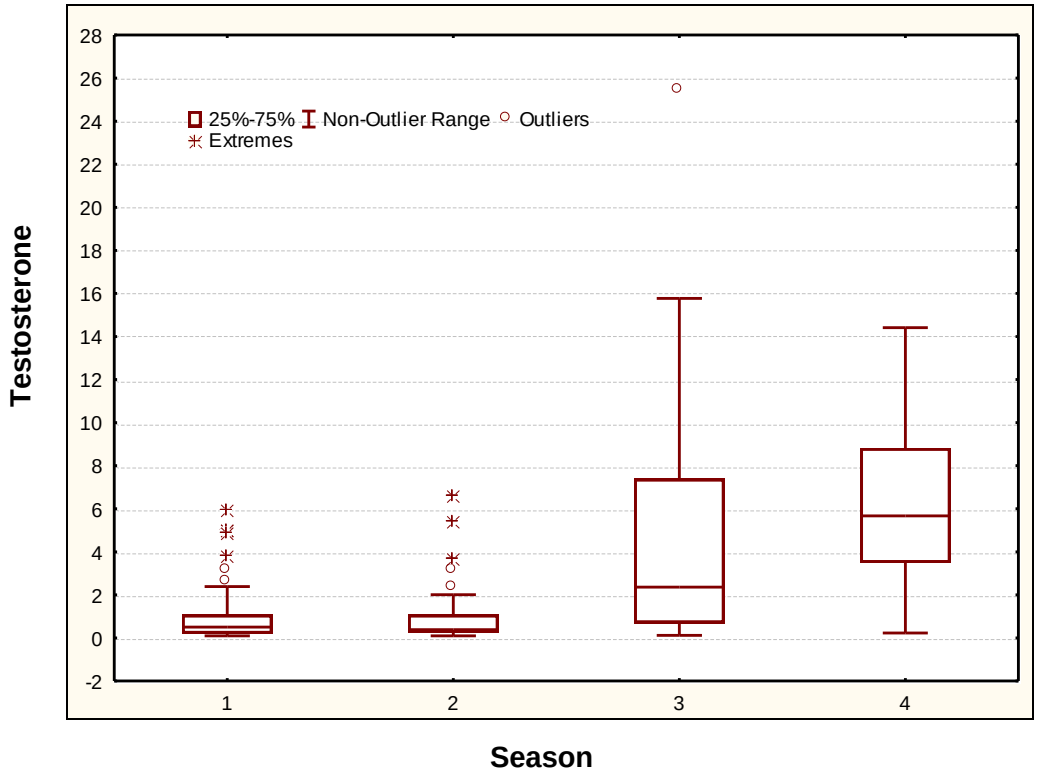

Figure 2. Box plot showing the testosterone seasonal mean levels of male Arbia goat.

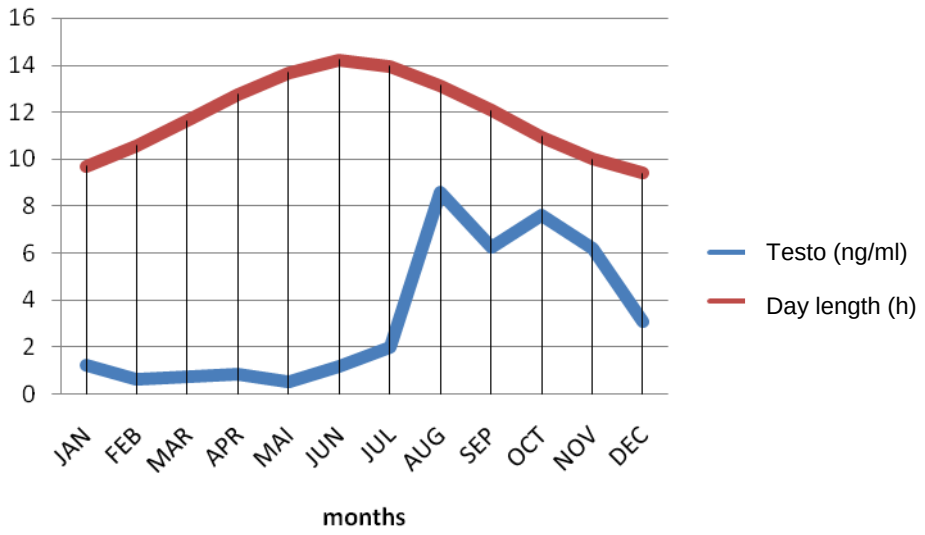

Figure 3. Comparison between plasma testosterone levels and photoperiod.

September) and a minimum in winter and spring (December-April) (Charallah et al., 2000). In seasonal breeds, the gonadotrope activity variations are responsible for the high seasonal variations of the sexual activity with alternate activity and inactive sexual periods. This effect is due to the photoperiodic variations which affect the central nervous system through the modification of the duration of nocturnal secretion melatonine (Chemineau and Delgadillo, 1994). The increase of the negative retroaction of the oestradiol in the hypothalamo-hypophysis axis is responsible for the weak gonadotrope activity during the anoestrus season. The frequency and the amplitude of the LH peaks and the testosterone concentration evolve with season. The testicular weight, the sexual behavior and the semen quality evolve at the same time as the hormonal modifications. The increase of the LH pulsatile activity (amplitude in June – July; frequency in September) lead to the beginning of the testicular increase (July- August) then the release of the testosterone (September) which stimulate the sexual behavior (increase in the number of mating, decrease in ejaculation latency) and the semen quality (October).

In our study, seasonal plasma testosterone patterns are similar to those reported by Todini et al. (2007), they found that the mean plasma testosterone concentration from all bucks of four Mediterranean breeds (Ionica, Garganica, Maltese and Red Syrian) were affected by season of sampling, being higher during summer than during autumn, which were in turn higher than during winter and spring.

Male north Moroccan goats show reproductive seasonality related to photoperiod. Testis measurements, sperm characteristics and plasma testosterone levels were low during winter, increasing through the spring and summer (Chentouf et al., 2011). In Zaraibi goats in Egypt, Barkawi et al. (2006) conclude that this breed has a distinct seasonal sexual activity. In this breed, libido, seminal characteristics and plasma testosterone concentration were highest during summer and lowest during spring. In this same breed, histological structure of the testis demonstrates a clear difference between seasons. The gonads are more active in summer and autumn compared to spring and winter. In the seminiferous tubules, the number of spermatic layers as the best indicator of testicular activity was high during autumn and low during spring (Eitedal et al., 2007). In vérata and Malguena bucks, plasma testosterone concentration was highest during autumn and summer when photoperiod is decreesing and lowest during winter and spring when photoperiod is increasing (Pérez et Mateos, 1995).

Hüseyin et al. (2011) shows that in Turkey, male white goats exhibit seasonal changes in plasma testosterone levels. The highest values were recorded in autumn and the lowest ones in spring. Zarazaga et al. (2009) studying effects of season and feeding levels on reproductive activity and semen quality in Payoya bucks goats conclude that this breed displayed considerable reproductive seasonality with intense sexual activity between August (mid- summer) and November (mid-late autumn). Al-Ghalban et al. (2004) report that in Damascus breed scrotal circumference begins to rise during spring and summer reaching maximal values in august. For local breeds in subtropical Mexico, plasma testosterone reach maximal levels in summer but the rise begins before or around the summer solstice (Delgadillo et al., 1999, 2002, 2004). Male Creole goats in subtropical northern Mexico displayed marked seasonal variations in testosterone secretion. However, low concentrations were observed from November to mid-June, and then the plasma testosterone concentration rose and remained elevated from July to October (Delgadillo et al., 2001).

In Mediterranean bucks the period of high testosterone

concentration started two months earlier than in breeds adapted to high latitude (Todini et al., 2007). These differences are most probably due to changes of photoperiod amplitude between subtropical and temperate zones. In fact in alpine bucks at 46°N plasma testosterone starts to increase in late August-September (Delgadillo and Chemineau, 1992). Compared to the onset of the breeding season in Creole bucks in subtropical Mexico with Alpine bucks at higher latitudes, it was suggested a shorter lag time between the perception of the photoperiodic signal and the expression of the physiological responses (Delgadillo et al., 2004).

In the Alpine bucks, the LH basic level (0.3 ng/ml of plasma), the frequency of pulses (around 1 in 8 h), their amplitude (less than 0.2 ng/ml) and so the LH mean concentrations (0.4 ng/ml of plasma) is low from January to May. The amplitude of pulses increases regularly in June and July to reach 1.0 ng/ml in August. Then in September, their frequency increases abruptly (3.5 pulses in 8 h). However, their amplitude decreases due to the inverse relation between frequency and amplitude and probably due also to the influence of the testosterone secreted in high quantity (4 ng/ml of plasma in August, 13 ng/ml in September). Following the high levels of LH and of the testosterone in August and September, a progressive decrease is noticed until January, then the annual cycle begins again (Saumande and Rouger, 1972; Delgadillo and Chemineau, 1992).

Similar observations were reported for the Australian cashmere bucks with a 6 month delay in the south hemisphere (Walkden-Brown, 1991). Goats in temperate latitude show important seasonal variations of their sexual activity in both male and female, the sexual activity is low between February and September. For Alpine and Saanen, testicular weight which is closely correlated to testicular spermatogenesis activity undergoes seasonal variation with low values from January to April and high values from September to December (Delgadillo et al., 1991). Volume ejaculation from Alpine and Poitevine bucks is high in autumn and winter (breeding season) and low in spring and summer (non breeding season) (Corteel et al., 1977). Walkden-Brown et al. (1994) report that sexual activity of Creole male goats of Guadeloupe did not vary throughout the year because in this area photoperiod is mainly constant.

The smelling of bucks is more pronounced during the breeding season than in the non-breeding season. This fact is due to the action of testosterone on the sebum glands in the skin, the head and the neck (Walkden-Brown 1991).

Conclusion

In conclusion, the results of this work support the hypothesis that native Arabia bucks studied displayed a clear seasonality of plasma testosterone levels, with an intense sexual activity in summer and autumn.

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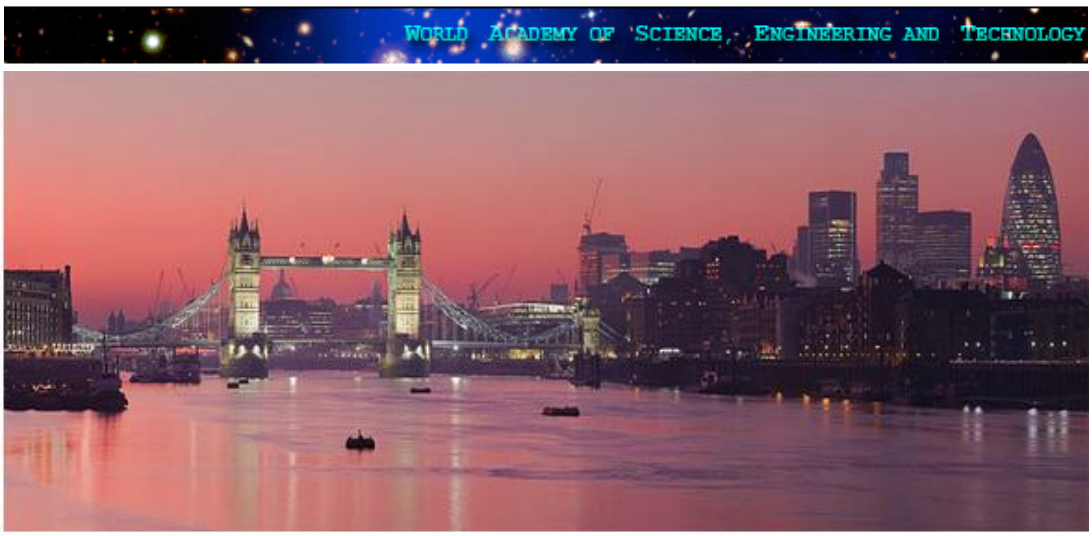

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