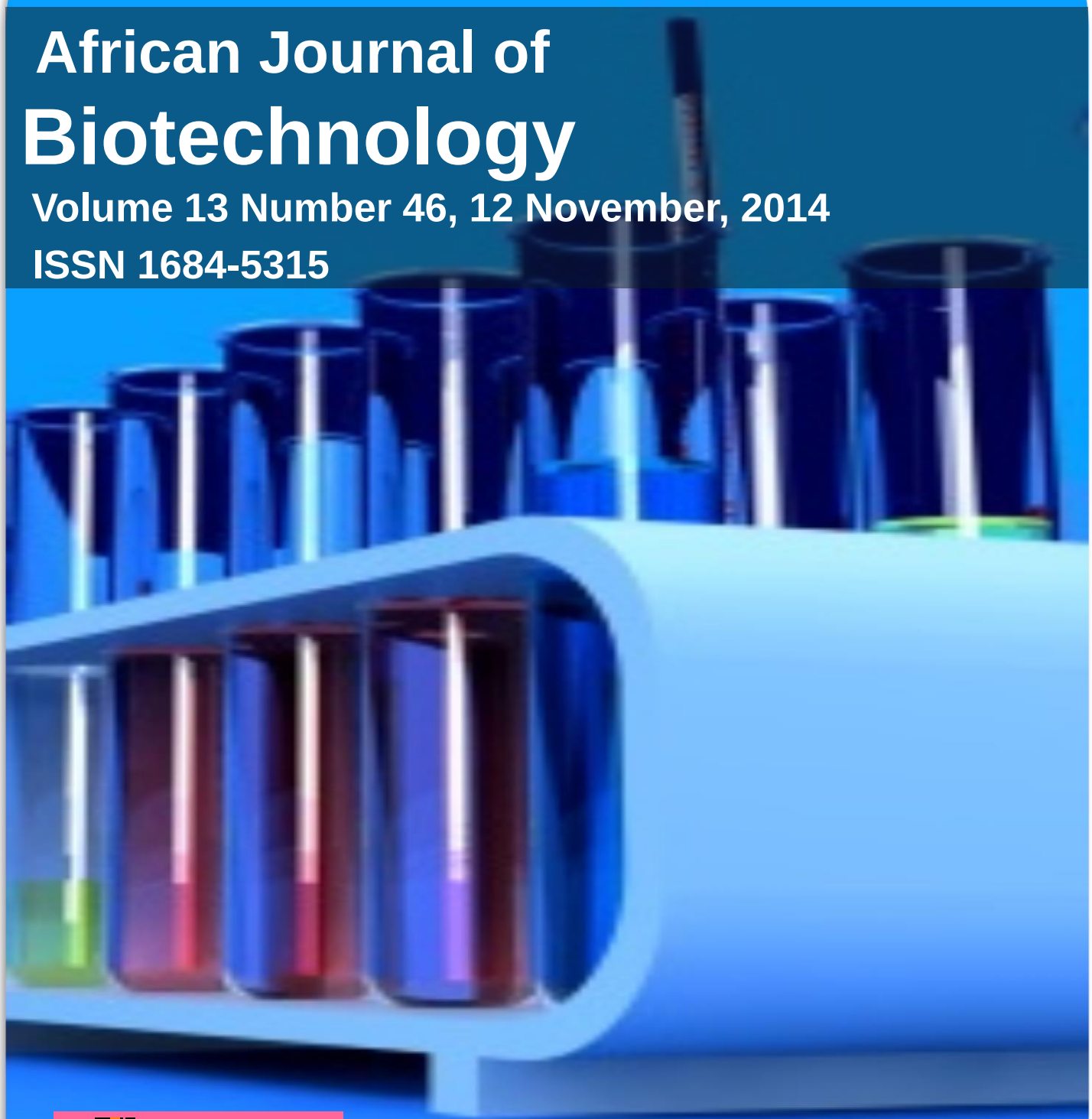
African Journal of Biotechnology

Volume 13 Number 46, 12 November, 2014
ISSN 1684-5315



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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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Vol. 13(46), pp. 4289-4293, 12 November, 2014
DOI: 10.5897/AJB2014.13690
Article Number: DEC886548562
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Nutritional characteristics of guava leaves and its effects on lipid metabolism in hypercholesterolemic rats

Juliana Mesquita Freire[1]*, Celeste Maria Patto de Abreu[1], Stella Maris da Silveira Duarte[2], Fernanda Borges Araújo de Paula[2], Adriene Ribeiro Lima[1], Rafaella Araújo Zambaldi Lima[1], Denise Alvarenga Rocha[1] and Anderson Assaid Simão[1]

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Received 3 February, 2014; Accepted 15 August, 2014

The objective of this study was to evaluate antioxidant substances, dietary fiber and antioxidant activity of guava leaves, and evaluate their amelioration hypercholesterolemia in rats. The leaves were dried and crushed into granulated powder. The active ingredient of the leaf powder was extracted by an ethanol/acetone mixture and called ethanol/acetone extract flour. Dietary fiber, vitamin C, beta-carotene, phenolic compounds and antioxidant activity were determined in the flours. Thirty (30) rats were used in the present study and categorized into: hypercholesterolemic control, non-hypercholesterolemic rats treated with ethanol/acetone extract flour, hypercholesterolemic rats treated with ethanol/acetone extract flour, non-hypercholesterolemic rats treated with leaf flours, and hypercholesterolemic rats treated with leaf flours. The animals were subjected to experimental hypercholesterolemia and treatment with flours for 42 days. At the end of the treatment, liver weight index *versus* body weight index, total hepatic lipids, C reactive protein, total cholesterol and fractions were evaluated. The treated hypercholeterolemic animals showed a reduction in total and fractionated cholesterol levels, but no difference was observed in the ratio liver weight index *versus* body weight index, total hepatic lipids and C reactive protein. It is concluded that guava leaves are a significant source of dietary fiber, phenolic compounds, vitamin C, beta-carotene, besides presenting antioxidant activity and hypocholesterolemic potential.

Key words: *Psidium guajava*, leaves, antioxidant, dietary fiber, hypocholesterolemia.

INTRODUCTION

Much of the world's population use various parts of plants in therapy for the control and prevention of diseases. Several people use the empirical knowledge about medicinal plants as the only therapeutic resource. In this context, plants that are popularly used for therapeutic purposes, but lack scientific evidence of their effects, are

of great value in the search for the development of new drugs of proven efficacy and safety (Balbino and Dias, 2010).

In recent decades, cardiovascular diseases have been the main cause of mortality in developed and developing countries. The increase in the incidence of those diseases is due to changes in nutritional patterns and physical inactivity, enabling an increase in the prevalence of hyperlipidemia, considered one of the main risk factors, due to the elevation of plasmatic levels of total cholesterol and fractions, associated to the decrease in high-density lipoprotein (HDL) level (Bruckner, 2008).

The excess cholesterol stimulates the production of reactive oxygen species, contributing to endothelial dysfunction, low-density lipoprotein (LDL) modification and reduction in the antioxidant defense system, such as the decrease in the activities of superoxide dismutase and glutathione peroxidase (Lester et al., 2009).

Many plants are used in the control and prevention of cardiovascular diseases. It is believed that its effect is due to the presence of antioxidants and dietary fiber, resulting in the inactivation of reactive species and cholesterol absorption, respectively (Beling et al., 2007).

Guava leaves have several antioxidant compounds, such as ferulic acid, quercetin, gallic acid, caffeic acid and ascorbic acid, which may play an important role in the body. Among its highlights are the following effects: hypoglycemic action, *in vitro* antioxidant activity, anti-inflammatory, antimicrobial, and hepatoprotective effects on hemostasis and blood coagulation (Jimenez et al., 2001; Thaipong et al., 2006; Gutiérrez et al., 2008; Deguchi and Miyazaki, 2010).

Thus, the objective of this study was to evaluate the contents of phenolic compounds, dietary fiber and anti-oxidant activity of the leaves of Pedro Sato guava, as well as its effect on plasma levels of total cholesterol and fractions, hepatic lipids and C-reactive protein.

MATERIALS AND METHODS

Sample collection and preparation

The leaves of Pedro Sato guava (*Psidium guajava*) were harvested, washed under running and distilled water and, soon afterwards, they were placed in forced-air ovens for drying for five days, at ±35°C. After drying, the leaves were ground in a Wiley type mill and the guava leaf flour was stored in hermetically sealed flasks under refrigeration. Then, ethanol/acetone (70/30, v/v) were added to the guava leaf flour during 24 h, followed by filtration. The supernatant was collected, submitted to evaporation and lyophilized (Rufino et al., 2007), and this new flour was called ethanol/acetone extract flour.

The leaves were identified by the College of Agriculture Lavras Herbarium, where the voucher specimen was deposited and received voucher number 26277.

Characterization of guava leaves

The content of soluble and insoluble dietary fiber was determined through the Sigma Total Dietary Fiber Kit. The results were expressed in g 100 g^{-1} sample (Association of Official Analytical Chemists - AOAC, 2005). Phenolic compounds were extracted with 50% methanol at a rate of 3 g sample diluted with 250 mL of 50% methanol at reflux for three consecutive times at 80°C and the extracts were combined, evaporated to 25 mL (Goldstein and Swain, 1963) and measured using the Folin-Denis reagent (AOAC, 2005). The results were expressed in mg of tannic acid g^{-1} dry material (DM).

The antioxidant activity was determined by the colorimetric methods 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Thaipong et al., 2006) and beta-carotene/linoleic acid (Rufino et al., 2007), using the extracts obtained for the determination of phenolic compounds. The vitamin C content was determined by the colorimetric method (Strohecker and Henning, 1967). The content of beta-carotene was determined by the colorimetric method (Nagata and Yamashita, 1992).

Biological analysis

The experiment was conducted according to the ethical principles for animal experimentation adopted by the Brazilian School of Animal Experimentation (COBEA), approved in 11/11/2010 by the Animal Research Ethics Committee of University Federal of Alfenas, protocol number 326/2010.

The experiment was conducted over six weeks, using 30 male Wistar rats (*Rattus norvegicus*), with initial body weight of 400.00 ± 50.00 g. The animals were kept in individual cages at 21°C, light/dark cycle of 12 h, with access to distilled water *ad libitum*. The rats were randomly divided into six groups with five rats each: NC: non-hypercholesterolemic control; HC: hypercholesterolemic control; NE: non-hypercholesterolemic rats treated with ethanol/acetone extract flour; HE: hypercholesterolemic rats treated with ethanol/acetone extract flour; NF: non-hypercholesterolemic rats treated with leaf flours; HF: hypercholesterolemic rats treated with leaf flours.

The rats in the non-hypercholesterolemic groups received a commercial diet (Biobase Bio-tec Ratos e Camundongos) during the six weeks of experiment. The rats in the hypercholesterolemic groups received the same diet, but with cholesterol (0.5%) and eolic acid (0.25%). For the preparation of the diet, the commercial food was triturated, and cholesterol and cholic acid were added. The mixture was moistened with water, shaped and taken to a ventilated oven at 35°C for two days (Rocha et al., 2012).

The guava leaf flour and ethanol/acetone extract flour was administered to the rats by gavage, once a day for 42 days, at a dosage of 50 mg Kg^{-1} body weight, while the control groups (non-hypercholesterolemic and hypercholesterolemic control) received water by the same administration method. Dosage choice was based on the study performed by Gutiérrez et al. (2008), who observed hepatoprotective, hypotensive, anti-inflammatory and

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Table 1. Chemical characterization and antioxidant activity of guava leaves.

Parameter	Leaf flour	Ethanol/acetone extract flour
Soluble dietary fiber (g 100 g^{-1} DM)	4.10 ± 0.39	ND
Insoluble dietary fiber (g 100 g^{-1} DM)	57.84 ± 1.23	ND
Vitamin C (mg 100 g^{-1} DM)	200.01 ± 6.96	ND
Beta-carotene (mg 100 g^{-1} DM)	12.80 ± 0.64	ND
Phenolic compounds (mg g^{-1} DM)	130.05 ± 6.71	369.93 ± 13.80
Antioxidant activity ($IC_{50\%}$ mg mL^{-1})	0.02 ± 0.00	0.01 ± 0.00
Antioxidant activity (inhibition %)	82.93 ± 0.99	82.81 ± 6.66

ND, Not detected. DM, dry matter.

analgesic effects.

At the beginning of the experiment and three days before euthanasia, the rats in the control groups (non-hypercholesterolemic and hypercholesterolemic control) were analyzed by puncture of the tail vein for total cholesterol, in order to confirm the induction of hypercholesterolemia.

At the end of the experiment, the rats remained under fasting for 12 h, and were then anesthetized with sodium thiopental (35 mg kg^{-1}). The blood was removed by the heart puncture technique, and the liver was dissected and separated.

Total and fractionated cholesterol levels were determined in the blood samples using the Labtest® enzymatic-colorimeter kit. Non-HDL cholesterol levels were determined by the difference between the total and HDL cholesterol levels. The ratio liver weight *versus* body weight was determined dividing the weight of the whole liver by the body weight of the animal. Hepatic lipids were determined by the methodology proposed by AOAC (2005). C-reactive protein levels were determined in the blood serum of the rats by the turbidimetric method using the Human® kit.

Statistical analysis

A completely randomized design in a 2 x 2 + 2 factorial outline was used; being two forms of extract preparation (leaf flour and ethanol/acetone extract flour), two diet types (hypercholesterolemic and non-hypercholesterolemic) and two additional treatments (hypercholesterolemic control and non-hypercholesterolemic control), totaling six treatments with five repetitions. The statistical analysis was conducted using the Sisvar program (Ferreira, 2000), and the means were compared by the Tukey test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Table 1 shows the contents of dietary fiber, antioxidant compounds and the antioxidant activity of guava leaf flour and ethanol/acetone extract flour. Guava leaf flour is shown as a good source of soluble (4.10 g 100 g^{-1} dry matter - DM) and insoluble fiber (57.84 g 100 g^{-1} DM). Dietary fiber was not detected in the ethanol/acetone extract flour. Dietary fiber prevents cholesterol absorption in the intestine, enhancing its excretion as bile salts, reducing therefore, cholesterol levels in the plasma (Rique et al., 2002; Mello and Laaksonen, 2009). The FDA recommends the consumption of 25 g dietary fiber per day on a 2,000-calorie diet; thus, guava leaves prepared in the form of flour are a rich source of this nutrient.

In the leaf flour, antioxidant substances such as vitamin C (200.01 mg 100 g^{-1} DM), beta-carotene (12.80 mg 100 g^{-1} DM) and phenolic compounds (130.05 mg 100 g^{-1} DM) were also found. On the other hand, only phenolic compounds (369.03 mg 100 g^{-1} DM) were reported for the ethanol/acetone extract flour, and these contents are higher than those found in guava leaf flour.

Beta-carotene acts as a lipophilic antioxidant and, together with vitamin C and phenolic compounds (hydrophilic antioxidants), it forms a strong defense against free radicals, by acting in different cell compartments.

Regarding antioxidant activity, guava leaf flour and the ethanol/acetone extract flour showed antioxidant activity by both methods, and the antioxidant activity of the ethanol/acetone extract flour was higher than that of the leaf flour by the DPPH method, and similar by the beta-carotene/linoleic acid method. These results can be explained by the higher contents of phenolic compounds found in the ethanol/acetone extract flour (Table 1), which resulted in a greater antioxidant activity by the DPPH method.

Several studies have shown that vegetables are sources of dietary fiber and antioxidants, and a diet rich in vegetables has a positive influence on plasma lipids and antioxidant activity (Leontowicz et al., 2001; Leontowicz et al., 2002; Salgado et al., 2008).

Biological analysis

Table 2 shows the contents of lipids, the liver weight/body weight ratio and the levels of C-reactive protein, with no statistical difference ($p \leq 0.05$) between the treatments for liver fat content and the liver weight/body weight ratio, when compared to their respective controls.

C-reactive protein is a protein synthesized by the liver in response to cytokines, which shows active inflammation. From Table 2, it is observed that there was

Table 2. Levels of hepatic lipids, liver weight/body weight ratio and C-reactive protein[1] in *Wistar* rats treated with guava leaves.

Treatment	Hepatic lipid (%)	Liver weight x body weight	PCR[1] (mg L^{-1})
NC[1]	3.56[b]	0.023[b]	0.2[c]
HC[2]	13.32[a]	0.030[a]	1.0[ab]
NE[3]	2.83[b]	0.023[b]	0.6[bc]
HE[4]	12.46[a]	0.030[a]	1.0[ab]
NF[5]	3.59[b]	0.025[b]	1.2[ab]
HF[6]	13.36[a]	0.031[a]	1.4[a]

Means followed by the same letter in the columns do not differ by the Tukey test (p≤0.05); [1]non-hypercholesterolemic control; [2]hypercholesterolemic control; [3]non-hypercholesterolemic rats treated with ethanol/acetone extract flour; [4]hypercholesterolemic rats treated with ethanol/acetone extract flour; [5]non-hypercholesterolemic rats treated with leaf flours; [6]hypercholesterolemic rats treated with leaf flours.

Table 3. Total serum cholesterol, triglycerides, HDL and non-HDL cholesterol in *Wistar* rats treated with guava leaves.

Treatment	Cholesterol (mg dL^{-1})	Triglyceride (mg dL^{-1})	HDL cholesterol (mg dL^{-1})	Non-HDL cholesterol (mg dL^{-1})
NC[1]	46.26[d]	40.06[ab]	14.98[b]	31.22[c]
HC[2]	96.70[a]	50.78[a]	8.80[c]	87.90[a]
NE[3]	50.40[cd]	38.14[b]	19.99[a]	30.90[c]
HE[4]	84.78[ab]	29.84[b]	16.10[ab]	70.04[b]
NF[5]	51.60[cd]	35.44[b]	17.01[ab]	32.22[c]
HF[6]	67.46[bc]	38.74[b]	13.01[bc]	54.28[b]

Means followed by the same letter in the columns do not differ by the Tukey test (p≤0.05); [1]non-hypercholesterolemic control; [2]hypercholesterolemic control; [3]non-hypercholesterolemic rats treated with ethanol/acetone extract flour; [4]hypercholesterolemic rats treated with ethanol/acetone extract flour; [5]non-hypercholesterolemic rats treated with leaf flours; [6]hypercholesterolemic rats treated with leaf flours.

a significant difference (p≤0.05) between the animals of the NC group with the HC group, that is, there was the formation of an inflammatory process that can progress to atherosclerosis, which demonstrates that none of the treatments was efficient in reducing the synthesis of C-reactive protein by the liver.

In Table 3, it is possible to observe that the HC group had serum levels of total cholesterol significantly (p≤0.05) higher than the ones of the NC group, indicating hypercholesterolemia. The values of the HC group (96.70 mg dL^{-1}) are close to those of other studies with rats that developed hypercholesterolemia (Machado et al., 2003; Rocha et al., 2012). Among the treatments, only guava leaf flour (HF group) decreased total cholesterol levels significantly (p≤0.05), compared to the HC group, with a reduction of 30.24%.

A hypercholesterolemic diet induces an increase in lipid accumulation in hepatocytes, leading to a fatty liver, which can be demonstrated in this study. The significant reduction in cholesterol levels provided in this study by guava leaf flour may be due to the presence of dietary fiber found in this flour (Table 1). Several studies show that soluble fibers cause a reduction in blood cholesterol levels. Some studies show that the reduction in serum cholesterol in rats was caused by soluble fibers that bind to cholesterol, leading to its elimination, thus reducing its absorption by the liver (Camire and Dougherty, 2003; Rocha et al., 2012).

For triglycerides, the results show that the treatments guava leaf flour (HF) and ethanol/acetone extract flour (HE) significantly (p≤0.05) decreased triglyceride levels, compared to the HC group, with a reduction of 41.24 and 23.71% for the HF and HE groups, respectively.

The highest reduction in triglyceride levels caused by the extract flour of guava leaves can be probably justified by the higher content of phenolic compounds and antioxidant activity shown in this extract.

For HDL cholesterol, only the HE group caused a significant (p≤0.05) increase in HDL levels, compared to the HC group, with an increase of 83.95%. The treatments guava leaf flour (HF) and ethanol/acetone extract flour (HE) significantly (p≤0.05) decreased the levels of non-HDL cholesterol, compared to the HC group, with a reduction of 38.25 and 20.32% for HF and HE groups, respectively.

The animals in the groups that received only the NE and NF commercial diet were statistically (p≤0.05), similar to the NC group in relation to serum levels of total choles-

terol, triglycerides, HDL and non-HDL cholesterol.

The reduction of non-HDL cholesterol is important, since several studies have shown that a reduction in total cholesterol is not an efficient measure to reduce cardiovascular mortality associated with cardiovascular disease patients or prevent their emergence (Liu et al., 2000; Magalhães et al., 2002).

Conclusion

Guava leaves (Pedro Sato cultivar) constitute a significant source of dietary fiber, phenolic compounds, vitamin C, beta-carotene, and they have antioxidant activity. These leaves present a hypocholesterolemic potential, since they cause a reduction in serum levels of total cholesterol, triglycerides and non-HDL cholesterol.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors would like to thank CAPES, CNPq and FAPEMIG for the financial support.

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academicJournals

Vol. 13(46), pp. 4294-4307, 12 November, 2014
DOI: 10.5897/AJB09.1157
Article Number: BC1D20E48565
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Production of fructosyltransferase by *Penicillium simplicissimum* in batch culture

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Received August 5, 2009; Accepted 5 June, 2013

Seventeen (17) strains of macro-fungi were screened for fructosyltransferase (FTase) production, of which *Penicillium simplicissimum* was selected for further work. FTase production was carried out in shake flask cultured at 30°C and 250 rpm for 48 h. For optimization of one factor-at-a-time method, effects of initial sucrose concentration, fermentation time, pH, inoculum size and agitation speed were studied on growth and extracellular FTase activity. These fermentation parameters were then optimized using response surface methodology (RSM) coupled with a central composite design (CCD). 20 g/l sucrose concentration, 36 h fermentation time, pH 6, 15 ml inoculum size and 150 rpm agitation speed were the optimum parameters obtained from RSM. The experimental values were in good agreement with predicted values within -0.057 to 0.023 errors. Nevertheless, the biomass and FTase (extracellular and intracellular) activities increased from 4.38 - 12.16 g/l, 92.76 - 99.46 IU/ml and 60.73 - 69.98 IU/ml in the unoptimized parameters to 14.16 g/l, 118.86 IU/ml (extracellular) and 71.98 IU/ml (intracellular) in the parameters optimized by RSM, respectively.

Key words: Fructosyltransferase, *Penicillium simplicissimum*, response surface methodology, fructooligosaccharides.

INTRODUCTION

Fructosyltransferase (FTase) are enzymes (E.C.2.4.1.9) that catalyzed the transfer of fructosyl residue from sucrose to another sucrose molecule or a growing fructose chain (Fernandez et al., 2007). FTase is produced by many fungal and bacterial strains (Ganie et al., 2014; Prapulla et al., 2000), where they are implicated in the biosynthesis of fructans (levan, inulin, graminant, phlein, etc.) (Fernandez et al., 2007). In microorganism, FTase is responsible for the production of fructooligosaccharides (FOS) (Antosova et al., 2008). FOS is extensively used in food and pharmaceutical industries because of their functional properties. It is present in some commonly consumed foods like fruits, vegetables, cereals and honey in trace amounts (Sangeetha et al., 2004a). However, the productions of FOS using FTase derived from microorganisms have attracted attention in the recent years (Yun et al., 1997). The enzymes were produced from a wide variety of micro-organisms such as *Aspergillus niger, Aspergillus phoenici* and *Aspergillus foetidus* (fungi), *Saccharomyces cerevisae* (yeasts), *Bacillus macerans* and *Streptococcus salivarius* (bacteria), respectively (Le Gorrec et al., 2002;

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Almeciga-Diaz et al., 2011; Han et al., 2011).

The cost of an enzyme is one of the main factors determining the economy of a process. Reducing the costs of enzyme production by optimization of the fermentation medium and process is the goal of basic research for industrial application. A group of researchers reported on the isolating and screening of micro-organisms for FTase production with higher activity (Fernandez et al., 2007; Yun et al., 1997; Le Gorrec et al., 2002), purifying and characterizing newly found enzymes (Prapulla et al., 2000; Sangeetha et al., 2004b; Lee et al., 1982; Heyer and Wendenburg, 2001). Antosova et al. (2008) separated fructosyltransferase from *Aureobasidium pullulans* and described its kinetic properties. Recently, Nemukula et al. (2009) isolated, purified and characterized fructosyltransferase from *Aspergillus aculeatus*. The influence of operating conditions on the synthesis of short chain fructo-oligosaccharides was also examined using random surface methodology. However, there is much less information available on optimization for FTase production. It is believed that with further optimization using a statistical tool such as response surface methodology (RSM), further increase in enzyme activity could be attained. It was reported that application of statistical experimental design techniques in fermentation process development could results in improved product yields and reduced process variability, development time and overall cost (Tari e al., 2007; Elibol, 1999).

The response surface methodology (RSM) is a powerful technique for testing multiple process variables because fewer experimental trials are needed. Conventional method of optimization involves varying one parameter at a time and keeping others constant. This method is extremely time consuming and often does not bring about the effect of interaction of various parameters (Elibol, 1999). The single dimensional search is laborious and less capable of reaching true optimum due to interactions among variables (Soni et al., 2007). In order to overcome this difficulty and determine the interaction between the studied variables, an experimental factorial design and response surface method were employed for the optimization process.

Penicillium simplicissimum, a strain isolated from the soil, had showed high galactosyltransferase activity when incubated in highly concentrated lactose solution (Cruz et al., 1999). Luonteri et al. (1998) previously reported that substrate specificities of three α-galactosidase of this strain (AGLI, AGLII and AGLIII) were determined by using various isolated galactose containing oligosaccharides and polymeric galacto(gluco)mannans. In fact this species had also been used to study the effect of glucose, ammonium, nitrate or phosphate limitation on the excretion of tricaboxylic acid (TCA) cycle intermediates in continuous system (Galmetzer and Burgsteller, 2002). In terms of lead and copper biosorption, *P. simplicissimum* was immobilized on loofa sponge in batch experiments (Li et al., 2008). Though *P. simplicissimum* had been utilized in various ways, to our knowledge, there is no scientific literature on statistical optimization for FTase production by this species. In this study, screening of selected micro- and macro-fungi for the production of FTase in shake flask culture were addressed. Optimization of growth (biomass) and FTase activities (intra- and extra-cellular) either using one-factor-at-a-time method or using a response surface methodological (RSM) approach were also described.

MATERIALS AND METHODS

Screening of isolates

Six strains of macro-fungi namely *Trametes lactinea, Trametes feei, Trametes pocas, Pycnoporous sanguineus, Lentinus sajor caju* and *Schizophyllum commune* and 11 strains of micro-fungi namely *Penicillium purpurogenum, Penicillium islandicum, P. simplicissimum, Penicillium rubrum, Aspergillus niger, Aspergillus terreus, Trichoderma viride, Penicillium clarifame, Penicillium variable, Penicillium spinulosum* and *Penicillium enopansum* were used in this study. All the tested strains were from the Forest Research Institute Malaysia (FRIM), Kepong, Selangor and University of Malaya, Kuala Lumpur, Malaysia. The strains were grown on potato dextrose agar and were maintained at 4°C.

Screening experiments were carried out in a rotary shaker using 250 ml unbaffled Erlenmeyer flask with 100 ml culture medium, comprised of (g/l): sucrose 10, malt extract 10 and NH_4Cl 1. The pH of the medium was adjusted to 5.6 prior sterilization. The flask were then inoculated with loopfuls of cells from the tested strains and incubated in the rotary shaker at 30°C, 250 rpm for 48 h. Samples were harvested and analyzed for biomass and FTase activities (extracellular and intracellular).

Microorganism and inoculum preparation

Fungal strain which was of higher FTase activity was chosen for subsequent studies. The strain was grown on potato dextrose agar at 20°C. The inoculum was prepared by transferring a loopful of spores from 3-day-old slant to 100 ml medium containing 1% (w/v) sucrose and 0.2% (w/v) yeast extract at pH 5.5 in 250 ml flasks, which were previously sterilized at 121°C for 20 min. The flasks were incubated at 33 ± 1°C on rotary shaker at 250 rpm for 24 h.

Production of FTase

Erlenmeyer flask (250 ml) composed of KH_2PO_4 (11 g/l), NH_4Cl (6 g/l) and yeast extract (10 g/l) were incubated in an orbital shaker according to the condition that resulted from the experimental design as in Table 1. After fermentation, the contents were filtered through Whatman filter paper (No. 1). The culture filtrate thus obtained was centrifuged at 8000 rpm for 20 min and the clear supernatant was used as enzyme sources.

Enzyme assay and analytical methods

Fructosyltransferase (FTase) activity was determined by measuring the release of reducing glucose in the reaction mixture as described by Sangeetha et al. (2004) with slight modification. 2.5 ml of 60 g/l sucrose was added to 0.5 ml of 0.1 M citrate buffer (pH 5.5) and 0.5 ml enzyme solution. The reaction mixture was incubated at 55°C for 1 h. The enzyme reaction was stopped by heating at 100°C for 15 min and the released glucose was measured using HPLC (CTO-

Table 1. Design of experiment levels of various parameters.

Variable	Coding	Unit	Level				
			-α	-1	0	+1	+α
Sucrose concentration	X_1	(g/l)	10	20	30	40	50
Fermentation time	X_2	h	12	24	36	48	72
pH	X_3		4	5	5.5	6	7
Inoculum size	X_4	(%w/v)	1	5	10	15	20
Agitation speed	X_5	rpm	100	150	200	250	300

10AS, Shimadzu, Japan) with refractive index detector using the polar bonded phase column (Kromasil LC-NH_2, 4.6 mm × 25 cm, 5 µm) at room temperature. The mobile phase was acetonitrile: water (75:25) at a flow rate of 1.0 ml/min. One fructosyltransferase unit is defined as the amount of enzyme activity required to produce one micromole of glucose per minute. The biomass was determined using dry cell weight method as described by Sangeetha et al. (2004b). The results were expressed as g/l.

Optimization by response surface methodology

Five experimental factors, sucrose concentration, initial pH, agitation speed, inoculum size and fermentation time were chosen for optimization of growth and enzyme production by the highest FTase producer. Response surface methodology using five factors and 5 level central composite designs were used to optimize the response of variables. Each factor in the design was studied at five different levels (-α, -1, 0, +1, +α) (Table 1). All variables were taken at a central coded value and considered as zero. The minimum and maximum ranges of variables were investigated and the full experimental plans with respect to their values in actual and coded form are listed in Table 1. The biomass and enzyme activity were studied as response. This criterion was used in all experimental designs and analyzed with the aid of Design Expert ver. 6.0.6 statistical software (Stat-Ease Inc, Minneapolis, MN). The biomass production and enzyme activity were analyzed using analysis of variance (ANOVA) combined with Fischer test to evaluate if a given term has significant effect ($p \leq 0.05$). The optimum levels of variables were obtained by graphical and numerical designs using Design expert program.

RESULTS AND DISCUSSION

Screening of fungal isolates for FTase production

Seventeen (17) fungal isolates from different families (*Trametes, Pycnoporous, Lentinus, Schizophyllum, Penicillium, Aspergillus* and *Trichoderma*) were screened for extra- and intracellular FTase activities and the results are shown in Figure 1. Under such cultivation conditions (pH 5, 30°C, 250 rpm for 48 h), FTase activities of all the strains ranged from 40 IU/ml (*Scyzophyllum commune*) to 506 IU/ml (*P. simplicissimum*). Most of the macro-fungi cultures were incubated longer because of a slower growth in potato dextrose agar (Figure 1a). The 24 h growth of the tested strain in sucrose submerged culture of shake flask showed that *P. simplicissimum* attained the highest growth at 3.8 g/L and 506 IU/ml for extracellular and 128 IU/ml for intracellular FTase activities, respectively.

Optimization using one factor at-a-time

Fermentation optimization by employing one factor at a time method involved changing one independent variable while fixing others at a certain level (Galmetzer and Burgsteller, 2002). Process parameters such as initial sucrose concentration, fermentation time, pH, inoculum size and agitation speed were included in this study.

Effects of initial sucrose concentration

The effects of different sucrose concentrations (10-50 g/l) on cell growth, extracellular and intracellular FTase activities were investigated (Figure 2a). The higher the sucrose concentration, the higher the biomass and FTase activities. Beyond 20 g/l of initial sucrose concentration, a reverse trend was observed. The maximum biomass and extracellular FTase were detected at 11.23 g/l and 99.46 IU/ml (Figure 2a), respectively. This may be attributed to higher sugar content and reduction in water activity as sucrose was a disaccharide, thus increasing its osmotic pressure and cause more substrate inhibition to the cells (Siqueira et al., 2008). This indicated that substrate concentration has a considerable effect on the distribution of fermentation products, as substrate utilization rates could be significantly influenced by the substrate concentration (Mu et al., 2006).

Effects of fermentation time

Figure 2b shows the fermentation profiles for growth and FTase activities over 72 h fermentation periods. After a lag period of 4 h, both biomass and FTase activities increased exponentially, reaching maximum at 12.16 g/l, 92.76 IU/ml (extracellular) and 69.98 IU/ml (intracellular), and later declined gradually till the end of the fermentation period. Kumar et al. (2008) reported that microbial enzyme reached its maximum production

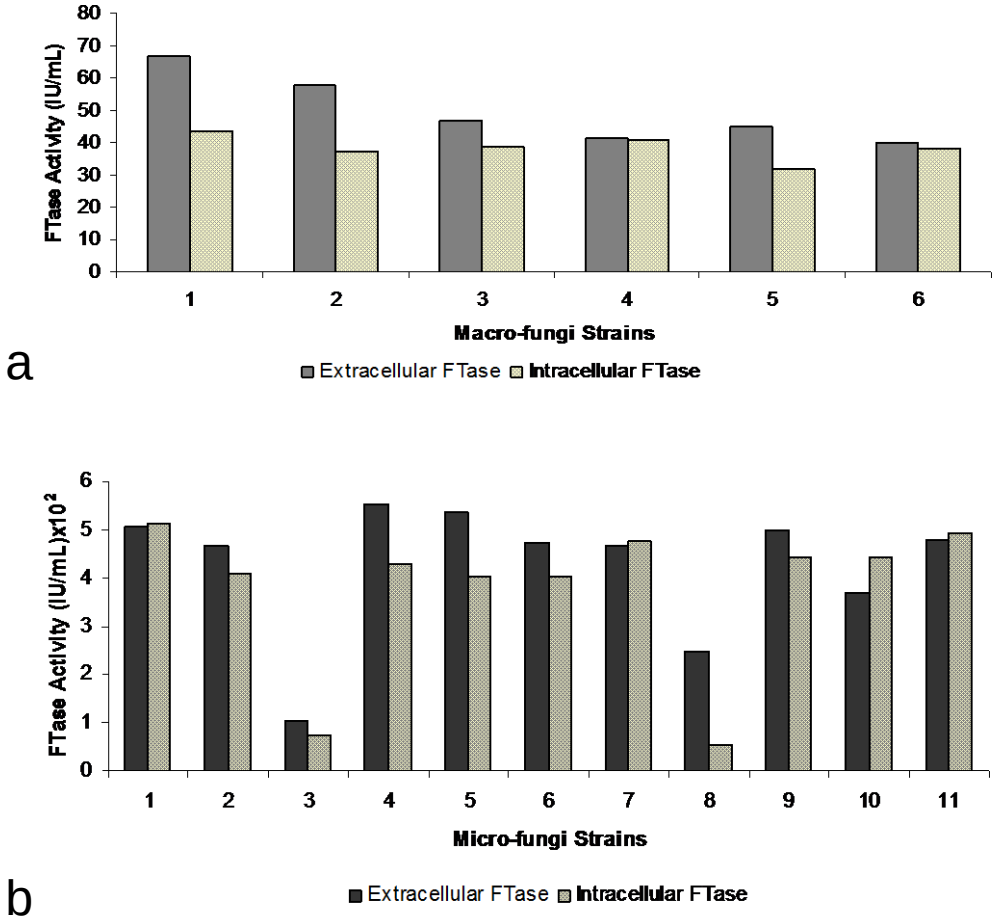


Figure 1. FTase activities by selected species of fungi in shake flask culture (a) Macro-fungi, 1 – *Trametes lactinea,* 2 – *Pycnoporous sanguines,* 3 – *Trametes pocas,* 4 – *Lentinus sajor caju,* 5 – *Trametes feei* and 6 – *Schizophyllum commune,* (b) micro-fungi, 1 – *Aspergillus niger,* 2 – *Aspergillus terreus,* 3 – *Trichoderma viride,* 4 – *Penicillium simplicissimum,* 5 – *Penicillium islandicum,* 6 – *Penicillium spinolosum,* 7 – *Penicillium variable*, 8 – *Penicillium rubrum*, 9 – *Penicillium enopasum*, 10 – *Penicillium clarifame* and 11 – *Penicillium purpurogenum*).

during log or late log phase (stationary phase). In stationary phase, nutrients might have been utilized mostly for cell multiplication and compensation of cell death (Anisha et al., 2008).

Effects of pH

FTase production is also influenced by the pH of the culture medium. To investigate the effect of pH on bioconversion of sucrose to glucose, it would be important to keep this parameter constant at the beginning of the fermentation period. Figure 2c shows the biomass and FTase activities profiles by *P. simplicissimum*. The highest FTase activity (extracellular) was observed at pH 6.0 which corresponded to 98.46 IU/ml after 24 h. At pH 4.0 to 5.5, both of the FTase activities were relatively low compared to pH 6.0. Similar effect of initial pH also occurred for the sucrose-to-glucose bioconversion by the FTase of *Aspergillus oryzae* CFR 202 in solid state fermentation using agricultural by-products (Sangeetha et al., 2004). FTase activity was significant only when the pH of the extract was in the pH range of 5-7. At acidic pH, the enzyme activity was less.

Effects of inoculum size

Inoculum concentration or size also played a crucial role in the determination of growth and enzyme production in submerged culture (Mukherjee et al., 2008). It is therefore important to select the suitable inoculum in respect to type, size and age to obtain a high yield of the desired product (Enshasy et al., 2000). As can be seen in Figure 3a, a lag phase of 4 h was observed for all biomass and enzyme activities. It then increased exponentially reaching the peak biomass of 9.16 g/l, extra- and

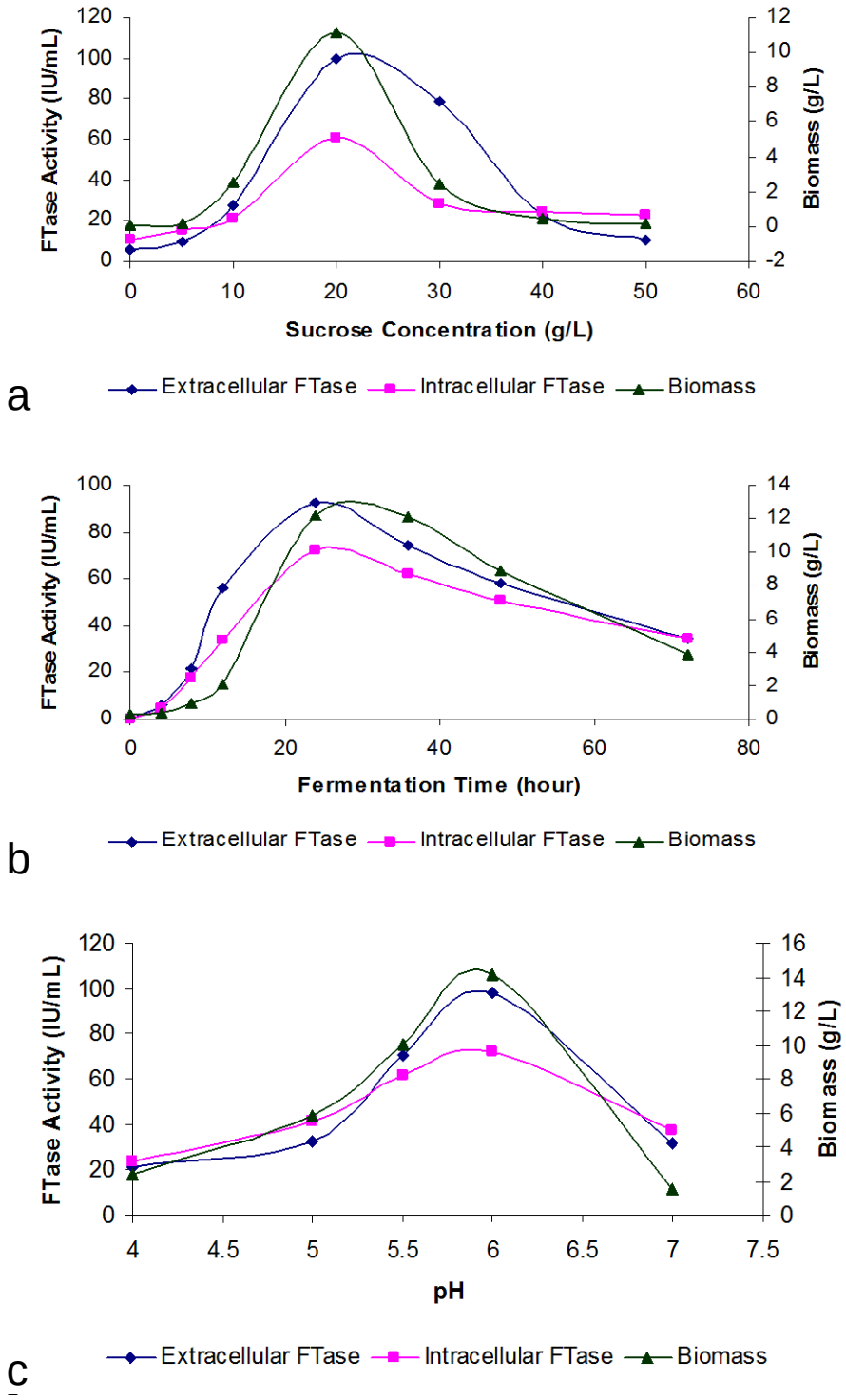
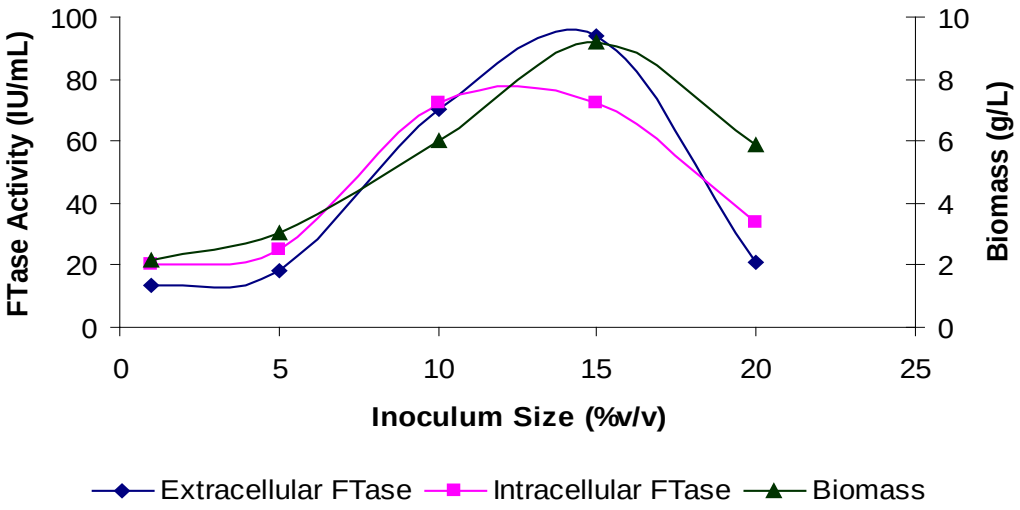


Figure 2. Fermentation profiles of biomass and enzyme activities at different (a) sucrose concentration, (b) fermentation time, and (c) pH.

intracellular FTase of 93.81 IU/ml and 65.98 IU/ml at 15% (v/v) inoculum concentration, respectively. Beyond this, a reverse trend was observed.

According to the study of Mukherjee et al. (2008), increase in inoculum size beyond 15% (w/v) resulted in steady decline of FTase activity and biomass which might be due to exhaustion of nutrients in the fermentation medium. In contrast, the lower inoculum size required longer time for the cells to multiply to a sufficient number for the maximum substrate utilization, thus resulting in



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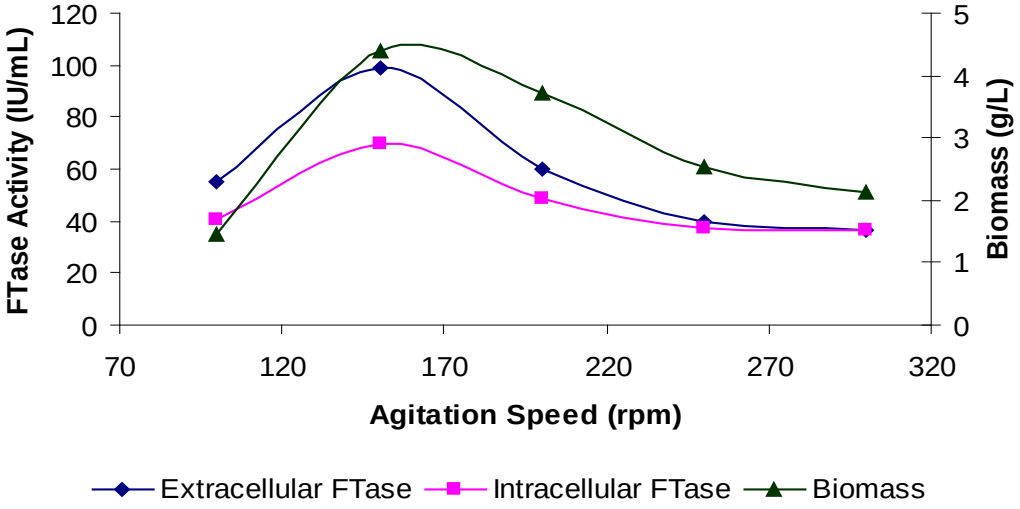

B

Figure 3. Fermentation profiles of biomass and enzyme activities at different (a) inoculums size, and (b) agitation speed.

lower enzyme yield (Ramachandran et al., 2004; Siqueira et al., 2008).

Effect of agitation speed

The effect of agitation on growth and FTase activities are shown in Figure 3b. An agitation of 150 rpm supported maximum biomass of 4.38 g/l and FTase activities of 98.79 IU/ml (extracellular) and 69.33 IU/ml (intracellular), respectively. Singh and Bhermi (2008) reported that at higher agitation speed increased the amount of dissolved oxygen and dispersion of macromolecules in the medium. It might contribute to the greater growth and enzyme production. However above 150 rpm, the biomass and enzyme activities tend to reduce gradually. This could be due to the shearing effect which was induced by the higher agitation speed on the cells and enzyme inactivation, thus contributing negatively towards cell growth and enzyme stability. According to the study of Wang et al. (2005), in many fungal fermentations, a higher agitation rate was necessary to provide adequate mixing and mass transfer, especially when the fungal cells grew in a freely dispersed form which resulted in a non-Newtonian broth and higher apparent viscosity. However, the mechanical forces induced by the rotating shaft could cause mycelia damages. Thus, agitation rate should be limited to a range that could avoid exerting

high shear stress on fungal mycelia.

Optimization using random surface methodology (RSM)

RSM is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariable equations simultaneously (Rao et al., 2000). It was used to optimize process parameters for the production of FTase by *Penicillium simplicissimum* in shake flask culture.

A central composite experimental design (CCD) for five independent variables was used to obtain the combination of values to optimize the response within the region of three dimensional observation spaces, which allowed one to design a minimal number of experiments (Tang et al., 2004).

Statistical experimental design

Table 2 shows the design matrix of the variables and the experimental results for growth and FTase activities of *P. simplicissimum*. The design independent variables and their range are classified in Table 1. Using multiple regression analysis on the experimental data, the following second-order polynomial equations were found to fit the extracellular FTase (Y_{EXT}), intracellular FTase (Y_{INT}) and biomass (Y_B), respectively.

$$Y_{EXT} = 28.63 - 0.75A - 2.69B + 4.96C + 5.32D - 1.91E + 6.68B^2 + 3.87C^2 + 1.42E^2 - 5.79AD - 3.02AE - 8.16BD + 3.84BE + 7.59CD - 2.41CE - 9.22DE \quad (1)$$

$$Y_{INT} = 31.58 + 0.48A - 0.79B - 0.97C + 0.95D + 1.22E - 1.51A^2 + 6.12B^2 - 1.88C^2 + 1.49D^2 - 1.57E^2 - 3.52AB - 2.22AC - 5.39AD + 4.99BD + 1.37CE - 2.67DE \quad (2)$$

$$Y_B = 7.63 + 1.19A + 1.84B - 0.05C + 0.06D + 1.81E - 0.54A^2 - 0.47B^2 - 0.53C^2 - 0.34D^2 - 0.69E^2 + 0.75AB + 1.67AE \quad (3)$$

Where, Y_{EXT} (IU/ml) and Y_{INT} (IU/ml) are the response factors for extracellular FTase, intracellular FTase and Y_B (g/l) for the cell biomass. *A, B, C, D and E* are values of independent factors for sucrose concentration (% w/v), fermentation time (h), pH, inoculum size (% v/v) and rate of agitation (rpm). In order to verify the validity of the models, it is necessary to conduct an analysis of variance (ANOVA) as presented in Table 3.

Table 3 shows that the regressions for biomass, extracellular and intracellular FTase models were significant (44.68, 47.10 and 33.21) and those lacked of fits were not significant (0.33, 1.25 and 0.63) at $p < 0.0001$ relative to pure error. The fit of the models were checked by the determination of coefficient, R^2. The R^2 value provided a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The R^2 value always lied between 0 and 1. The closer the R^2 value to 1.00, the stronger the model was and the better it predicted the response. In this case, the value of R^2 for biomass, extra- and intra-cellular FTase were 0.942, 0.954 and 0.942, respectively. These values showed that 5.8, 4.6 and 5.8% of the total variables were not explain by the models. The '*Pred* R^2' of 0.8934 and 0.8669 for extracellular and intracellular FTase production and 0.8677 for biomass production were in reasonable agreement with the '*Adj* R^2' of 0.9388, 0.9132 and 0.9026. This indicated a good agreement between the experimental and predicted values for FTase and biomass production. The adjusted R^2 corrected the R^2 value for the sample size and for the number of terms in the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than the R^2. This should be a caution signal as too many terms were present in the model (Haaland, 1989). The plot of predicted versus experimental extracellular FTase activity is shown in Figure 4, with R^2 = 0.954, thus indicating an excellent adequacy of the proposed model.

Interaction of parameters

The three-dimensional response surface curves were plotted to study the interaction among different parameters to find out the optimum condition for extracellular FTase activities. Intracellular FTase activity was not explained as the trend of the response surface plot was similar to extracellular FTase. Figure 5a shows the response of extracellular FTase with respect to initial sucrose concentration and agitation. An increase in agitation speed from 200-300 rpm with initial sucrose concentration 10-20 g/l induced the extracellular FTase activity to 35.15 IU/ml. However, the enzyme activity tends to reduce as the concentration of sucrose increased to 60 g/l though the agitation speed was higher (300 rpm). According to the study of Chen and Liu (1996), sucrose was the best inducer for β-fructofuranosidase (FFase) or β-D-fructosyltransferase (FTase) production by *A. japonicas* TIT-90076 and enzyme yield was suppressed at initial sucrose concentrations greater than 25%. It is well known that with increase in the sucrose concentration, the osmotic pressure also increased, thus eventually increasing the percentage of cell fractures (Quan et al., 2008). Previous study also indicated that some microorganisms grew best in medium that has slightly lower osmotic concentration than its own because this facilitated the diffusion of nutrients into the cells (Okafor, 2007). According to the study of Sangeetha et al. (2004b), enzyme activity decreased at higher sugar concentration. The predicted R2 values of extracellular and intracellular FTase production and biomass were in reasonable agreement with the adjusted R2. This indicated a good agreement

Table 2. Central composite design (CCD) of factors in coded levels with extracellular, intracellular FTase enzyme activities and biomass concentration as response.

Run	Coded Factor Level					FTase activity (Extracellular) (IU/ml)		FTase activity (Intracellular) (IU/ml)		Biomass (g/L)	
	Sucrose Concentration (g/L)	Fermentation Time (h)	pH	Inoculum Size (%w/v)	Agitation (rpm)	Actual value	Predicted value	Actual value	Predicted value	Actual value	Predicted value
	X1	X2	X3	X4	X5						
1	1	1	-1	-1	1	56.304	59.673	24.158	25.897	1.643	2.892
2	0	0	2.378	0	0	63.105	43.126	47.670	49.112	0.460	0.188
3	-1	-1	1	-1	-1	13.405	17.370	23.952	21.392	4.035	4.609
4	-1	1	-1	1	1	32.037	27.934	29.562	30.507	3.916	4.516
5	0	0	0	0	-2.378	45.475	39.590	24.508	25.657	3.003	3.566
6	-1	1	-1	-1	-1	18.638	21.026	37.411	40.010	0.218	0.337
7	-1	1	1	-1	1	47.203	45.557	25.258	21.152	5.070	4.858
8	-1	-1	1	1	1	58.473	62.258	20.653	21.405	4.163	4.616
9	0	0	0	0	0	24.625	28.630	35.034	33.928	1.704	2.304
10	-1	1	-1	1	-1	24.908	30.199	36.498	35.598	0.114	0.648
11	-1	-1	-1	1	1	43.254	41.971	49.983	49.370	5.052	4.697
12	1	-1	1	1	1	43.331	43.126	33.788	36.940	5.875	6.028
13	-1	1	1	1	1	47.532	48.221	28.463	33.687	2.461	2.436
14	0	0	0	0	0	24.649	28.630	22.646	26.496	0.503	0.632
15	-1	-1	-1	-1	-1	20.117	17.783	50.743	49.129	3.916	4.404
16	1	-1	-1	-1	-1	31.900	33.901	30.181	27.838	5.639	5.586
17	0	0	0	0	0	30.014	28.630	36.426	30.932	3.830	3.099
18	-1	1	1	-1	-1	18.299	20.614	55.272	54.147	6.265	6.692
19	-1	1	1	1	-1	55.004	60.153	24.613	26.427	5.268	5.524
20	1	-1	1	1	-1	78.987	82.501	36.418	35.542	12.313	12.104
21	1	-1	-1	1	-1	49.940	52.547	34.277	36.161	2.723	3.349
22	0	0	-2.378	0	0	39.597	38.696	48.330	50.514	7.066	6.794
23	-2.378	0	0	0	0	27.255	30.423	27.094	31.656	5.983	5.350
24	0	0	0	0	0	33.757	28.630	35.061	31.910	12.153	11.782
25	2.378	0	0	0	0	22.447	26.838	25.104	28.289	2.398	2.380
26	-1	-1	-1	1	-1	58.063	59.596	29.534	29.959	7.470	7.398
27	0	0	0	0	0	34.099	28.630	41.568	43.731	5.355	5.481
28	0	0	0	0	2.378	29.715	33.925	31.456	31.302	6.591	8.006
29	1	1	1	-1	-1	32.369	36.731	35.767	33.518	2.838	2.089

Table 2. Contd.

Run	Coded factor Level					FTase activity (Extracellular) (IU/ml)		FTase activity (Intracellular) (IU/ml)		Biomass (g/L)	
	Sucrose Concentration (g/L)	Fermentation Time (h)	pH	Inoculum Size (%w/v)	Agitation (rpm)	Actual value	Predicted value	Actual value	Predicted value	Actual value	Predicted value
	X1	X2	X3	X4	X5						
30	0	0	0	2.378	0	36.816	41.301	26.689	26.327	7.254	6.959
31	0	-2.378	0	0	0	70.295	72.820	51.272	48.960	4.366	4.765
32	1	-1	1	-1	1	32.848	30.989	24.877	27.669	13.470	12.622
33	1	1	1	-1	1	49.313	49.592	21.257	21.883	2.514	1.743
34	1	-1	-1	-1	1	43.253	41.069	26.722	24.170	6.853	7.423
35	-1	1	-1	-1	1	53.717	55.638	25.627	25.572	1.044	0.575
36	-1	-1	1	-1	1	33.127	26.954	62.335	64.313	9.084	9.352
37	0	0	0	0	0	33.842	28.630	22.761	23.232	5.356	4.733
38	1	1	-1	1	-1	20.811	23.149	21.023	18.626	4.084	4.506
39	1	1	1	1	1	27.666	29.089	37.186	37.773	6.191	5.575
40	1	-1	1	-1	-1	31.192	33.488	44.794	42.280	5.459	5.875
41	1	1	1	1	-1	57.200	53.104	21.657	19.786	1.010	0.606
42	-1	-1	1	1	-1	118.860	98.760	71.978	68.074	14.164	13.486
43	1	1	-1	1	1	10.369	8.802	34.164	31.582	8.883	7.634
44	0	2.378	0	0	0	64.184	59.983	29.520	31.582	4.514	7.634
45	-1	-1	-1	-1	1	33.130	37.034	35.748	31.582	8.289	7.634
46	1	-1	-1	1	1	26.456	22.839	30.374	31.582	7.111	7.634
47	0	0	0	-2.378	0	13.568	15.960	30.624	31.582	8.289	7.634
48	0	0	0	0	0	32.653	28.630	24.106	31.582	7.999	7.634
49	0	0	0	0	0	34.291	28.630	33.634	31.582	8.614	7.634
50	1	1	-1	-1	-1	42.521	37.144	35.752	31.582	7.508	7.634

between the experimental and predicted values for FTase and biomass production. The adjusted R^2 corrected the R^2 value for the sample size and for the number of terms in the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than the R^2. This should be a caution signal as too many terms were present in the model (Haaland, 1989). The plot of predicted versus experimental extracellular FTase activity is shown in Figure 4, with R^2 = 0.954, thus indicating an excellent adequacy of the proposed model.

Interaction of parameters

The three-dimensional response surface curves were plotted to study the interaction among different parameters to find out the optimum condition for extracellular FTase activities. Intracellular FTase activity was not explained as the trend of the response surface plot was similar to extracellular FTase. Figure 5a shows the response of extracellular FTase with respect to initial sucrose concentration and agitation. An

Table 3. ANOVA for response surface quadratic model.

Source of variations	Sums of squares	Degrees of freedom	Mean square	*F*-value	Significance (*P* value)
Extracellular (Y_{EXT})					
Regression	14569.53	15	971.30	47.10	<0.0001
Residual	701.10	34	20.62		
Pure Error	120.79	7	17.26		
Lack of Fit	580.31	27	21.49	1.25	0.4079
Total	18068.5	49			
Intracellular (Y_{INT})					
Regression	5779.02	16	361.19	33.21	<0.0001
Residual	358.86	33	10.87		
Pure Error	107.94	7	15.42		
Lack of Fit	250.92	26	15.42		
Total	6137.88	49	9.65	0.63	0.8196
Biomass (Y_B)					
Regression	515.04	13	39.62	44.68	<0.0001
Residual	31.92	36	0.89		
Pure Error	13.53	7	1.93		
Lack of Fit	18.39	29	0.63	0.33	0.9843
Total	546.96	49			

Extracellular: R^2, 0.9541; adjusted R^2, 0.9338; predicted R^2, 0.8934; Intracellular: R^2, 0.9415; adjusted R^2, 0.9132; predicted R^2, 0.8699; Biomass: R^2, 0.9416; adjusted R^2, 0.9026; predicted R^2, 0.8677.

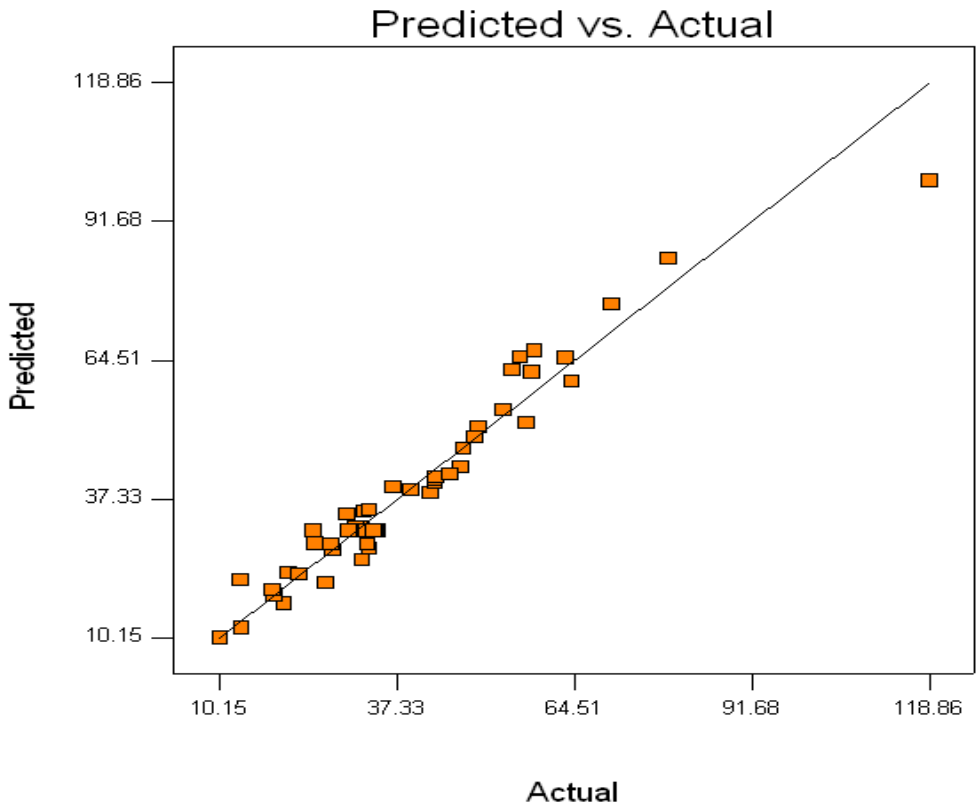

Figure 4. Plot of predicted versus experimental for extracellular FTase activity.

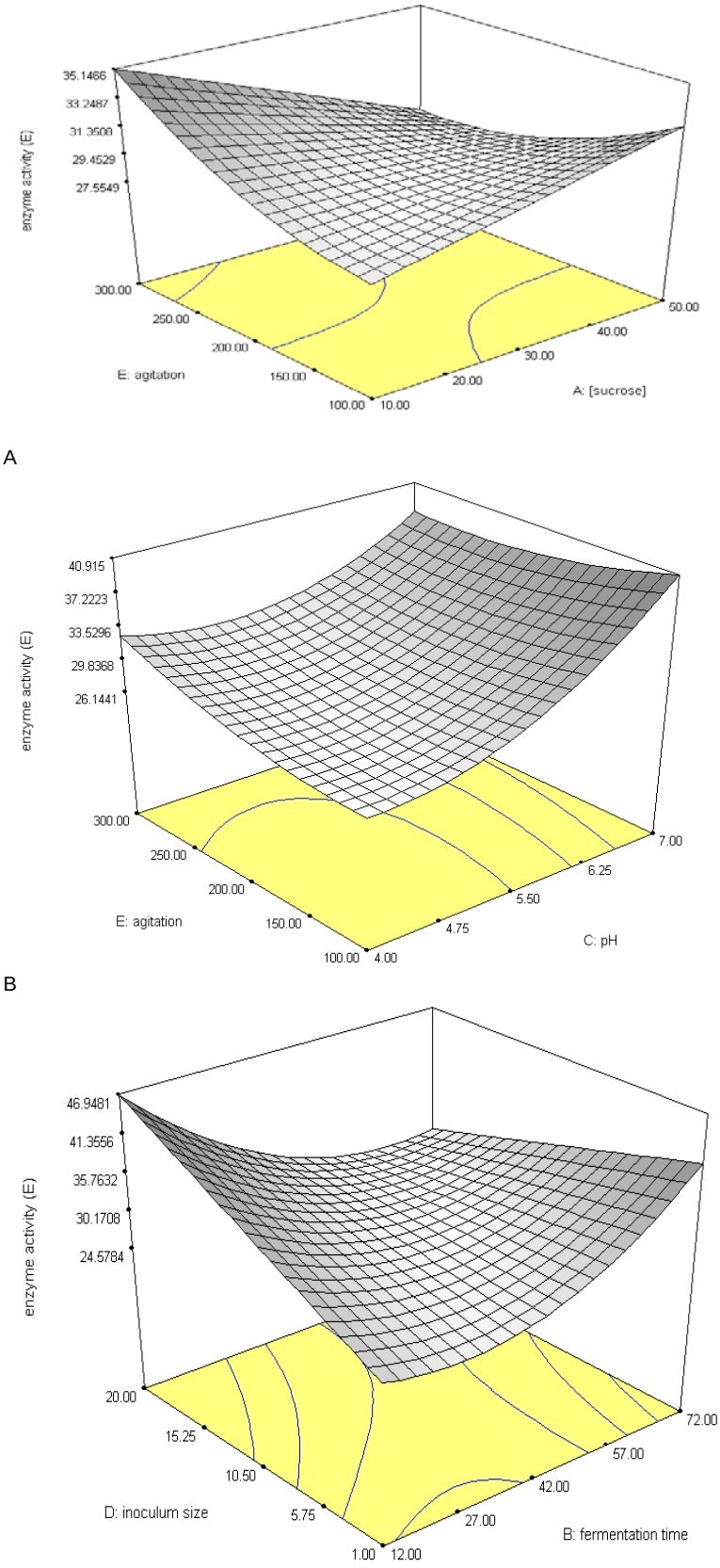

Figure 5. Interaction of fermentation parameters between (a) initial sucrose concentration and agitation, (b) pH and agitation, and (c) fermentation time and inoculum size,

increase in agitation speed from 200-300 rpm with initial sucrose concentration 10-20 g/l induced the extracellular FTase activity to 35.15 IU/ml. However, the enzyme activity tends to reduce as the concentration of sucrose increased to 60 g/l though the agitation speed was higher (300 rpm). According to the study of Chen and Liu (1996), sucrose was the best inducer for β-fructofuranosidase (FFase) or β-D-fructosyltransferase (FTase) production by *A. japonicas* TIT-90076 and enzyme yield was suppressed at initial sucrose concentrations greater than 25%. It is well known that with increase in the sucrose concentration, the osmotic pressure also increased, thus eventually increasing the percentage of cell fractures (Quan et al., 2008). Previous study also indicated that some microorganisms grew best in medium that has slightly lower osmotic concentration than its own because this facilitated the diffusion of nutrients into the cells (Okafor, 2007). According to the study of Sangeetha et al. (2004b), maximum enzyme activity decreased at higher sugar concentration. At lower sucrose concentration, the reaction proceed faster thus producing more FTase (Mukherjee et al., 2008). This is in agreement with the results of other researcher (Tucker and Thomas, 1994; Antosova et al., 2008) who used *Aspergillus japonicas*, *Aureobasidium pollulans* and *Aspergillus niger* ATCC 20611 as their tested strain using sucrose as a substrate.

For the effects of agitation on extracellular FTase (Figure 5a), the higher the agitation speed, the higher the FTase activity. According to the study of Lee and Chen (1997), enzymes seemed to be affected by higher agitation speed as it may increase shearing effects into the cells. Moreover, the higher the agitation speed increased, the higher the amount of dissolve oxygen and dispersion of macromolecules in the medium. It might therefore have contributed to greater growth and enzyme production (Seth and Chand, 2000). However, the shearing effect induced by the higher agitation speed on the cells and enzyme inactivation may sometimes contributed negatively towards cell growth and enzyme stability (Seth and Chand, 2000; Lejeune and Baron, 1995). In fact, similar result can also be observed in this study.

In Figure 5b, an inclined form of three dimensional plot inferred that interaction between pH and agitations were evident with significant linear correlations. Higher extracellular FTase activity obtained at pH 6.25-7.00, is fairly similar to that of FTase or FFase from *Arthrobacter* enzyme (pH 6.5-6.8) but different from FTase or FFase from *A. japonicas*, *A. niger*, *Penicillium* and *Aureobasidium* (pH 5.0-6.0) (Lee et al., 1982; Chen and Liu, 1996; Lee and Chen, 1997; Sangeetha et al., 2004b). Generally, the effect of pH on microbial growth may be attributed to the hydrogen (H^+) ion concentration. H^+ can be considered as substrate under the pH range of 6.0 - 7.0 but act as inhibitor under acidic and alkaline pH. Additionally, the enzyme has almost no transfructosylating activity below pH 3.0 or above pH 10.0 (Sautour et al., 2003).

Inoculum size is another notable feature that influenced the FTase activities. As can be seen in Figure 5c, two interaction options were observed, in which, higher extracellular FTase activity were detected either at higher inoculums size with short fermentation time or lower inoculums size with longer fermentation time. Sangeetha et al. (2004b) reported that an optimal inoculum size in the fermentation process should be provided, as lower inoculum density may give insufficient biomass, whereas a higher inoculum density may produce too much biomass which depleted the nutrients necessary for the product formation. Generally, enzyme production attains its peak when sufficient nutrients were available to the biomass. Conditions with a misbalance between nutrients and proliferating biomass resulted in decreased enzyme synthesis (Shafiq and Haq, 2002). In other words, the critical inoculums level apparently depended on spore preparation, germinability, conditions during germination and medium composition (Tucker and Thomas, 1994). Similar results were also obtained in the present study, whereby, the highest FTase activity was 93.712 IU/ml at higher inoculums size within 12 h of the fermentation period. At lower inoculums size (1 % w/v), the highest FTase activities at 13.568 IU/ml was detected after 72 h. A decline in enzyme activity after 72 h of fermentation could be either due to decrease in nutrient availability in the medium, catabolic repression of the enzyme or the lag time. Antasova et al. (2002) reported that maximum FTase production was achieved on the 98 h, however in this study, the highest FTase activity was detected after 12 h of the fermentation period. This is more significant as compared to previous researchers as reduction of cultivation time reduced the cost of FTase production.

Validation of the models

In order to validate the adequacy of the model equations (Equations 1, 2 and 3), a total of five verification experiments for each response (extracellular, intracellular and biomass) were carried out under various fermentation conditions as presented in Table 4. The validation data were separately analyzed using the Design Expert ver. 6.0.6 statistical software (Stat-Ease Inc, Minneapolis, MN). The correlation coefficients (R^2) between the experimental and predicted values were 0.954 and 0.942 for extracellular and intracellular FTase and 0.942 for cell biomass, with a significant level of $p < 0.0001$. The results of analysis indicated that the experimental values are in agreement with the predicted ones, and also suggested that the models of the equations were satisfactory and accurate.

Conclusions

To date, no reports are available in literature regarding

Table 4. Validation of the data and models constructed.

Run	A	B	C	D	E	FTase activity (extracellular)			FTase activity (intracellular)			Biomass concentration		
						Exp	Predicted	Error (ε)	Exp	Predicted	Error (ε)	Exp	Predicted	Error (ε)
1	20	48	6	15	150	107.96	98.76	-0.093	71.978	68.074	-0.057	14.164	12.788	-0.107
2	20	48	6	15	150	101.46	98.76	-0.027	69.867	68.074	-0.026	13.212	12.788	-0.033
3	20	48	6	15	150	97.912	98.76	0.009	70.565	68.074	-0.036	11.452	12.788	0.104
4	20	48	6	15	150	99.873	98.76	-0.011	68.792	68.074	-0.011	12.854	12.788	0.005
5	20	48	6	15	150	98.888	98.76	-0.013	69.243	68.074	-0.017	12.482	12.788	0.023

the optimization of fermentation parameters for FTase production using RSM by P. simplicissimum. Therefore, this study will serve as a base line of the initial studies in this field. Through this optimization, the optimal conditions for maximum FTase activity were sucrose 20 (w/v), fermentation time of 36 h, pH 6, inoculum size of 15% (w/v) and 150 rpm rate of agitation. The developed parameters showed higher extracellular FTase activity of 118.86 IU/ml as compared to unoptimized parameters at 92.76 - 99.46 IU/ml. It should be noted here that the activities were obtained from crude enzyme that had not been purified and strain which did not go through any strain improvement step.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This research was supported by Science Fund Grant (02-01-05-SF0100) by the Ministry of Science, Technology and Innovation (MOSTI), Malaysia. The authors would like to thank Dr. Noorlidah Ahmad from the University of Malaya and Dr Salmiah Ujang from Forest Research Institute Malaysia (FRIM) for the kind supply of the strains.

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Vol. 13(46), pp. 4308-4319, 12 November, 2014
DOI: 10.5897/AJB2014.13849
Article Number: 621CF8D48567
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Analysis of genetic diversity and population structure among exotic sugarcane (*Saccharum* spp.) cultivars in Ethiopia using simple sequence repeats (SSR) molecular markers

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Received 10 April, 2014; Accepted 10 June, 2014

The present study was performed to study genetic relationships and population differentiation of 90 introduced sugarcane accessions in Ethiopia by means of 22 SSR molecular markers. The 22 SSR markers amplified a total of 260 alleles, of which 230 were polymorphic with a mean of 10.45 alleles per SSR locus. The range in allele number was 4–22. A high level of polymorphism with a mean of 60.51% polymorphic loci within the genotypes was detected. The polymorphic information content (PIC) ranged from 0.231 to 0.375 with an average of 0.303. Measures of effective number of alleles and genetic diversity on average were 1.55 and 0.317, respectively, across all the 22 markers evaluated. The SSR genetic profiles obtained using the 22 markers enabled complete discrimination among all the 90 introduced sugarcane cultivars. The neighbor- joining unweighted pair group method with arithmetic mean (UPGMA) cluster analysis based on the simple matching dissimilarity indices unambiguously distinguished all sugarcane genotypes with three major clusters and 11 groups. The same clustering pattern was also found in the PCoA analysis. In all the geographical populations, genotypes from the same country were often in different clusters and likewise accessions from different countries often clustered together indicating the possibility of exchange of materials between countries. Population genetic differentiation showed F_{st} values among pairs of populations ranging from 0.0024 to 0.5134 with an overall average of 0.0590. The average gene flow (Nm) among populations was 1.7213. Nei's unbiased genetic distance ranged from 0.018 to 0.279 with an overall average of 0.053. Genetic identity values were in the range of 0.756 to 0.992 with overall average of 0.950. The genetic relationship information of the cultivars will help sugarcane breeders to select the appropriate parents in their breeding programs to maximize yield as well as to maintain genetic diversity.

Key words: Sugarcane, Ethiopia, simple sequence repeats (SSR), genetic diversity, population genetic differentiation.

INTRODUCTION

Sugarcane is an important food crop of the tropics and sub tropics that is cultivated in about 74 countries between 40°N and 32° 5´S (Anonymous, 1998). A wide variety of sweet cane types existed from which were

selected thick barrelled, high sucrose soft sticks and this crude method of selection eventually produced better cane types. Sugarcane produces numerous valuable by-products like alcohol used by pharmaceutical industry, ethanol used as a fuel, bagasse used for manufacturing paper and chipboard and press mud used as a rich source of organic matter and nutrients for crop production (Muhammad and Farooq, 2007). The genome of modern sugarcane cultivars is a complex blend of aneuploidy and polyploidy derived from the interspecific hybridization involving different *Saccharum* species particularly, *S. officinarum* and *S. spontaneum* (Swarup et al., 2009). Use of an efficient molecular marker system is essential for sugarcane genome for understanding the genetic and taxonomic complexity, and broadening the genetic base of sugarcane cultivars, thereby improving sugar yield and its stabilization against abiotic and biotic stresses.

Since the start of the sugar industry, Ethiopia has been relying on importation of sugarcane varieties from many source countries to satisfy the varietal requirements of the sugarcane plantations. So far more than 300 sugarcane varieties have been imported and preserved in germplasm conservation garden located at Wonji. Importing variety *per se* is not an easy task, besides this all introduced varieties might not become successful commercial cultivars. For instance, among these introduced varieties, currently only 6-7 varieties are grown widely and commercially for sugar production in the three old sugar estates namely Wonji, Metehara and Fincha and the recent ones like Tendaho, Kesem, Beles, Kuraz and Wolkayit. These varieties are of very old generation and are contracted with many problems and not satisfactorily adapted to the different sugarcane growing areas of the country. This could be because of the reason that the varieties were released to suit the growing conditions of the country of source. At present, in Ethiopia sugarcane plantations are increasing at large and the demand for new superior varieties for each sugarcane growing region is expected to rise than ever. Accordingly, preparation to launch sugarcane breeding program in the country is underway to generate high yielding varieties that can adapt to different agro ecologies of sugarcane plantations and withstand different biotic and abiotic stresses. As the first step in this endeavour to broaden the genetic base of germplasm, exploration and collection of local sugarcane germplasm growing in the country in the back yards of small holder farmers since ancient times has been conducted and more than 200 clones were collected and preserved (Esayas et al., 2012).

There is lack of information on the imported varieties vis a vis pedigree, identity of the varieties etc., which is very difficult to trace as many of the clones are of old generation and significant number are of unknown sources. In spite of a long history of introduction, no systematic effort has been made to understand the genetic relationship of these cultivars. For better use of these materials in the breeding program and for broadening the genetic base characterization of these germplasm, efficient molecular marker is a must. The modern genetic breeding requires crosses between productive and genetically divergent parents, in order to have better heterotic effect and variability in the segregant generations (Cruz, 2001).

Genomic microsatellite markers are capable of revealing high degree of polymorphism. Sugarcane (*Saccharum* spp.), having a complex polyploid genome requires such informative markers for various applications in genetics and breeding. Microsatellite markers have gained considerable importance in plant genetics and breeding owing to their many desirable genetic attributes including hyper variability, wide genomic distribution, co-dominant inheritance, reproducibility, multi-allelic nature, and chromosome specific location (Singh et al., 2010). These markers are amenable to high throughput genotyping and are thus suitable for paternity determination, construction of high density genome maps, mapping of useful genes, marker-assisted selection, and for establishing genetic and evolutionary relationships (Swarup et al., 2009). SSRs are an ideal means for the identification of the genetic constitution of modern sugarcane cultivars of inter-specific origins (Giovanni et al., 2003).

Hence, this study was conducted to quantify genetic variability/diversity among introduced sugarcane germplasm collections using SSR markers and to assess genetic associations within and among populations of clones from different source countries.

MATERIALS AND METHODS

Plant materials

A total of 90 accessions of sugarcane introduced from a range of sugarcane producing countries around the world were used in this study. These materials were selected amongst more than 300 introduced sugarcane varieties found at the field conservation garden of Sugar Corporation of Ethiopia, Research and Training located at Wonji (Table 1). Selection was made taking into consideration the variation in place of origin that is source countries and different periods of introductions to the country. For genomic DNA isolation, young immature leaves of sugarcane genotypes was taken and stored in zip lock plastic bags containing Grade 12, blue silica gel. The silica was added in the ratio 1:5 which was sufficient enough to dry the leaves completely. This way, the leaf samples were transported to Sugarcane Breeding Institute, Coimbatore, India and total DNA isolation was conducted using the method described by Walbot (1988) in the laboratory of department of biotechnology between November 2012 and February 2013.

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Table 1. Introduced sugarcane varieties in Ethiopia used for SSR diversity analysis.

Number	Cultivar	Country of origin	Year of introduction	Number	Cultivar	Country of origin	Year of introduction	Number	Cultivar	Country of origin	Year of introduction
1	B3172	Barbados	1983	17	CB 41-76	Campos (Brazil)	1970	33	CP 36/111	Do	1970
2	B37172	Do	1956	18	C 105-73	Do	1974	34	CP44/101	Do	1957
3	B 4098	Do	1960	19	CB 56-20	Do	1983	35	CP 48/103	Do	1960
4	B41211	Do	1970	20	CO 245	Coimbatore(India)	1970	36	CP 52/68	Do	1974
5	B 4425	Do	1974	21	CO 331	Do	1954	37	H48/4605	Hawaii	1965
6	B 45154	Do	1957	22	CO 434	Do	1970	38	H49/5	Do	1965
7	B 49119	Do	1962	23	CO 440	Do	1963	39	H49/3533	Do	1974
8	B 5364	Do	1965	24	C120-78	Do	1970	40	CP 65/357	Canal point	1983
9	B 62347	Do	1974	25	CO 475	Do	1956	41	CP 8/1026	Do	1984
10	BO 10	Bihar-Orissa (India)	1960	26	CO 513	Do	1960	42	CP 1/441	Do	1983
11	BO 14	Do	1974	27	CO 740	Do	1962	43	CP 71/443	Do	1984
12	BO 29	Do	1974	28	CO 957	Do	1965	44	COS 109	Unknown	1965
13	BO 3	Do	1970	29	CO 1208	Do	1987	45	COS 443	Do	1965
14	CB 36-14	Campos,(brazil)	1974	30	CP 29/116	Canal point	1953	46	COS 510	Do	1962
15	CB 38-22	Do	1959	31	CP 29/291	Do	1954	47	COK 30	Do	1970
16	CB 40-35	Do	1983	32	CP 36/105	Do	1959	48	D 42/58	Demerara, (Guyana)	1974
49	D 141/46	Demerara, (Guyana)	1974	63	H 39/3633	Hawaii	1960	77	N 14	Natal (South Africa)	1980
50	D 188/56	Do	1974	64	H 44/3098	Do	1960	78	N 50/93	Do	1965
51	DB 95/57	(Demerara) X Barbados	1974	65	L 60-14	Louisiana, USA	1974	79	WD II	Do	1953
52	DB 377/60	Do	1974	66	L 60-25	Do	1974	80	C86-12	Cuba	2003
53	DB 386/60	Do	1974	67	L 60-35	Do	Unknown	81	C132-81	Do	2003
54	DB 414/60	Do	1974	68	L 60-40	Do	1974	82	NCD 310	Natal	1953
55	DB 414/66	Do	1983	69	M 31/45	Mauritius	1957	83	NCD 349	Do	1970
56	Ebene 1/37	Unknown	1957	70	M 53/263	Do	Unknown	84	PR 905	Puerto Rico	1959
57	E 88/56	Do	1974	71	M 112/34	Do	1960	85	PR 980	Do	1965
58	E 188/53	Do	1974	72	M 147/44	Do	1957	86	PR 1000	Do	1960
59	E 188/56	Do	1974	73	Mex 52/29	Mexico	1970	87	PR 1007	Do	1970
60	F 134	Formosa, (Taiwan)	1970	74	Mex 59/1828	Do	1983	88	PR 1059	Do	1974
61	F 141	Do	1970	75	N 6	Natal (South Africa)	1983	89	PPQK 1604	Do	1958
62	H 38/4443	Hawaii	1960	76	N 11	Do	1987	90	Pindar	Unknown	1957

DNA isolation

Approximately 500 mg leaves of each genotype were separately ground to fine powder in liquid nitrogen using pre-cooled sterilized mortar and pestle. Fine powder was transferred into sterilized centrifuge tube containing 10-15 ml of grinding buffer and centrifuged at 6500 rpm for 6 min at 4°C. After discarding the supernatant, the pellet was suspended in 3 ml of suspension buffer and 200 µl of 20%

SDS was added, mixed gently and placed on a water bath at 70°C for 15 min. After incubation, 1.5 ml ammonium acetate (7.5 M) was added, mixed well and placed on ice for half an hour. The sample was centrifuged at 14,000 rpm for 10 min at 4°C. Then supernatant was decanted in 6 ml iso-propanol to precipitate the DNA. After incubation for 30 min in ice the sample was centrifuged at 15,000 rpm for 15 min at 4°C to pellet the DNA, supernatant was discarded and the pellet was resuspended in 500 µl TE and kept in water bath at 65°C for 15 min. The DNA solution was transferred into sterilized microfuge tube using sterilized cut tips and 15 µl RNase (10 mg/ml) was added and incubated in a water bath at 37°C for 15 min. Then equal volume of choloroform:iso-amyl alcohol (24:1) was added and followed by centrifugation at 10,000 rpm for 10 min at room temperature. The aqueous phase was pipetted out and transferred into fresh microfuge tube. This was repeated two times. Then DNA was precipitated by adding 50 µl sodium acetate ($1/10^{th}$) and 1 ml of ice-cold absolute ethanol and kept at -20°C for half an hour. After this, the DNA was pelleted by centrifuging at 15000 rpm for 15 min at 4°C. The pellet was washed two times with 70% ice-cold ethanol. Finally, the pellet was dried in air and dissolved in 200 µl TE and stored at -20°C until used.

Assessment of quality and quantity of extracted DNA samples

The quantity and quality of the extracted genomic DNA were checked and normalization or adjustments to 10 ng μl^{-1} was done on agarose gel (0.8%) using lambda DNA standard (Thermo Scientific, USA). The DNA was quantified by loading the samples on 0.8% agarose gel containing 0.5 $\mu g\ ml^{-1}$ of ethidium bromide. The DNA was normalized to 10 ng μl^{-1} concentration by comparing visually the diluted DNA samples with the standard λ DNA molecular weight markers on 0.8% agarose gel by running it in 0.5xTBE (Tris-borate EDTA) buffer at a constant voltage (180 V/125 mili amp) for 45 min. The images of gels were documented under UV illumination using Uvi -Tech gel documentation system (DOL-008.XD, England).

SSR assays

Twenty two (22) SSR markers designed in laboratory of the Department of Biotechnology, Sugarcane Breeding Institute, Coimbatore, India were used (Table 2). PCR reactions were performed in 15 µl reaction mixtures in 96-well PCR plates (Thermocycler, MJ Research Inc Model: PTC-100). Each PCR reaction contained 10 ng of genomic DNA (3 µl), 10XTaq Buffer with $MgCl_2$ (1.5 µl), 1.25 mM dNTPS (2.4 µl), each 30 ng (1 µl) forward and reverse primers, 3 U/µl Taq DNA polymerase (0.3 µl) and sterile 5.8 µl milliQ water. PCR amplification was carried out at 94°C for 5 min initial denaturation, followed by 30 cycles of 94°C (denaturation) for 1 min, 50-65°C annealing temperature (depending on AT and CG content of the primers) for 1 min and 72°C extension for 1 min. After completion of all 30 cycles, a final extension of 10 min at 72°C was performed.

PAGE

Checking of the amplification of the PCR products was done on 1% agarose gel containing 0.5 ml/10 ml ethidium bromide (10 mg ml^{-1}) with 10 base-pair DNA ladder by running it at a constant voltage of 180 V/125 mili amp for 45 min. The amplification was visualized under UV illumination using Uvi Tech gel documentation system (DOL-008.XD, England). After this, PCR products were loaded on 4% PAGE gels, polyacrylamide: bis acrylamide (29:1) and electrophoresed in 1x TBE buffer at constant power of 180 V/125 milli amp) for 1 h using Sequi Gen® GT nucleic acid electrophoresis cell and the products were resolved using silver staining procedure.

Data analysis

Only clear and unambiguous bands of SSR markers were scored. No assumption on the genetic nature of the alleles was made due to the polyploid nature of sugarcane and the absence of a segregation analysis (Gillet, 1991). Hence, each allele was scored in a dominant manner and transformed into either a 0 (absent) or 1 (present) matrix. Although SSRs are classified as co-dominant type markers, they have been treated as dominant markers in this study.

Genetic diversity analysis

The software program Genalex 6.5 (Peakall and Smouse, 2006, 2012) was used to calculate parameters such as: genetic distance, number of different alleles (NA), number of effective alleles (NE), Shannon's information index, Analysis of Molecular Variance, etc. Genetic associations were determined with Darwin V. 5.0 software (Perrier et al., 2003) using neighbour-joining coefficient. Popgene program (Yeh et al., 1999) was also used to determine genetic diversity, polymorphic loci, gene flow and F-values. Finally, the basic statistics such as polymorphic information content (PIC) and gene diversity were estimated using PowerMarker v. 3.25 (Liu and Muse, 2005).

RESULTS AND DISCUSSION

Allelic diversity

The genotyping of a total of 90 introduced sugarcane varieties, 81 of them from fourteen countries around the world and the remaining 9 from unknown sources (Table 1) with 22 SSR markers were allowed to identify a total of 260 alleles (Table 2). However, variations in the total number of fragments and the number of polymorphic fragments were evident. The data for microsatellite loci diversity are summarized in Table 3. Out of the total of 260 alleles amplified, 230 were polymorphic with a mean of 10.45 alleles per SSR locus (Table 2).

The highest level of polymorphism was detected in SOMS173 (100%) followed by SOMS167 (98.89%), SOMS169 (98.89%), and SOMS29 (97.78%). The range in allele number was 4–22, with the marker SOMS147 having the highest number of alleles (22), followed by SOMS173, SOGL50, SOMS29 and SOGL38 with 21, 17, 16 and 15 alleles, respectively (Table 2). Fragments size ranged from 100 (SOGL50 and SOMS173) to 1700 bp (SOGL41) in length. The polymorphism information content (PIC) calculated as a relative measure of informativeness for each of the markers ranged from 0.231 for the markers SOGL41 to 0.375 for the marker SOMS166 with an average of 0.303. Number of different alleles was in the range of 1.09 in SOMS166 to 2.0 in SOMS173 with overall mean of 1.75. Measure of effective number of alleles on average was 1.55 across all the 22 markers evaluated. The markers with the highest levels of effective number of alleles (1.71-1.82) were SOMS29, SOMS167, SOGL11 and

Table 2. Details of SSR markers with their annealing temperature (Ta), sequences (forward and reverse), range of product size and total number of amplified bands and total number of polymorphic bands given by the primer are shown in bracket.

Primer name	Forward (5'- 3')	Reverse (3' - 5')	T_a (^{0}C)	Range of Product Size (bp)	Number of bands
SOMS167	AGCAGAGACACACGCACA	ACAAGAGGAGGTTCAGGG	54	180-900	10(10)
SOMS166	GTCTCTTCTCCAGTTCTCCTT	GTCTTCTCCACAACCACCT	50	400-950	4(2)
SOMS148	GATGACTCCTTGTGGTGG	CTTGACGACCCTGCTGCT	54	120-700	4(3)
SOMS168	ATGGCGTCTCGTCTCGTT	ACCTCAGTCTTGTCTTCCTTC	50	130-1400	10(10)
SOMS88	AGATGGATGAGGGTTTCTTT	CCTACGAGTTTATTCTTCAGT	55	260-540	10(9)
SOMS169	ACAGCACAGGCTCTCTCTT	TCCTTTCAGGCATCCATC	52	170-1200	13(13)
SOMS96	AACTTGACCCTTCTTCTTCC	GCCGATGGACACCTTGAC	55	540-780	5(3)
SOMS119	CAACATCTCACGAAACAATAC	AACACCTCCTACACTGACACA	55	350-850	10(8)
SOMS109	ATCCTTTGTCGTCTCCGT	AGTTGGGTGTGTATTTGGTG	54	280-780	9(8)
SOMS68	AACTGAAGCAGCACCAACT	TTGTCTAATACCCTGACCTGA	56	175-1200	14(12)
SOMS158	ATAATGACTGAACCTCTCCC	CTTCCTGTGCTTCCTGGT	54	260-690	9(7)
SOMS29	ACCAGTTCCTCTACGCCC	CATCCCATCCCTTGTGTC	55	165-650	16(16)
SOMS143	TGACTTGGAATAACACAAAGAA	ATGGGATGGATAATAAGCAGT	54	230-400	14(13)
SOMS147	AGCGAACCCTAATGGAGA	GGGAGACATCGTAGACCTG	55	240-820	22(20)
SOGL11	GTGCTGAATGAGAGAGTGGT	TCCAGGTCGCTGTAAGAA	55	320-600	8(8)
SOGL37	TTCTCTGACTTCCAATCCAA	ATCAAGCACGCCCGCCTC	55	290-500	7(6)
SOGL15	CATCAGTATCATTTCATCTTGG	CAGTCACAGTCGGGTAGA	56	250-450	11(10)
SOGL36	TCCTCATTACCATTTGTTCC	CCTCCTCTTGCTGGACTT	52	280-1000	14(12)
SOGL50	GCTACTATGGACAACAGGG	ATGAAGAGACGAGACGAAGA	55	100-450	18(17)
SOGL38	AAGCAAGCAAGGCAAACT	GTGGGCAACGCACTGGTC	50	180-840	16(15)
SOGL41	TGAGGACGGGATGAAGAC	CGGTTACTGTTTGAGGGAG	52	210-1700	15(9)
SOMS173	GTGGACGAGAAGTGGAAGT	ATAGGAGGGCAGGACAAG	54	100-1200	21(21)

SOMS168 whereas; the markers SOMS 96 and SOGL41 had the lowest value, 1.26 (Table 3). The highest Shannon's information index 0.63 was recorded for marker SOMS29 while the lowest value 0.26 was observed in SOMS96 with mean of 0.47 across markers. The highest gene diversity 0.440 was observed using the marker SOMS29 while the lowest value of 0.167 was observed with SOMS96 with an overall mean of 0.317.

Genetic analysis has been hindered in sugarcane due to lack of sufficiently informative markers. Less information is available about the genetic diversity within and between *Saccharum* cultivars which has been based mainly on morphological characteristic.

This study reveals considerable SSR polymorphisms with a mean of 60.51% polymorphic loci within the genotypes under study (Table 5). High levels of polymorphism were detected with an average number of 10.45 polymorphic fragments per primer pair (Tables 2). Every marker was able to amplify varying numbers of DNA fragments (bands) from all 90 clones, regardless of their geographical origins. Chen et al. (2009) reported SSR polymorphisms when studying on sugarcane cultivars from breeding programs in China and other countries. High degree of polymorphism had been also reported by Singh et al. (2010) using SSR markers on sugarcane species and commercial hybrids in India.

Genetic relatedness among accessions

Estimated genetic dissimilarities (Dice's dissimilarity) of the germplasm were visualized in radial

Table 3. Number of different alleles (N_a), number of effective alleles (N_e), Shannon index (I), gene diversity and PIC of the 22 SSR markers used in the study.

Locus	Sample size	N_a	N_e	I	Gene diversity	PIC
SOMS167	90	1.98	1.77	0.60	0.418	0.334
SOMS166	90	1.09	1.35	0.27	0.188	0.375
SOMS168	90	1.98	1.71	0.59	0.406	0.257
SOGL37	90	1.73	1.60	0.48	0.330	0.348
SOGL15	90	1.89	1.58	0.51	0.340	0.307
SOGL36	90	1.69	1.46	0.42	0.274	0.324
SOGL50	90	1.86	1.67	0.55	0.380	0.307
SOMS88	90	1.87	1.69	0.54	0.373	0.296
SOMS169	90	1.98	1.56	0.50	0.332	0.327
SOMS96	90	1.10	1.26	0.26	0.167	0.241
SOMS119	90	1.78	1.42	0.40	0.260	0.337
SOMS109	90	1.71	1.37	0.37	0.236	0.281
SOMS68	90	1.69	1.48	0.43	0.282	0.329
SOMS158	90	1.53	1.56	0.46	0.318	0.261
SOGL38	90	1.91	1.63	0.54	0.362	0.296
SOGL41	90	1.27	1.26	0.28	0.175	0.231
SOMS29	90	1.96	1.82	0.63	0.440	0.317
SOMS143	90	1.84	1.35	0.38	0.237	0.220
SOMS147	90	1.78	1.68	0.54	0.372	0.331
SOMS148	90	1.81	1.64	0.51	0.354	0.338
SOMS173	90	2.00	1.53	0.45	0.299	0.346
SOGL11	90	1.97	1.77	0.60	0.421	0.272
Mean	90	1.75	1.55	0.47	0.317	0.303

diagram (Figure 1). Analysis of the SSR data using Dice's dissimilarity indices showed that pair wise genetic dissimilarity (GD) values ranged from 6.1 (between PR1007 and Ebene 1/37) to 73.5% (between CB41-76 and H38/4443) with a mean of 38.74% (data not shown). In the dendrogram, clones CB41-76 and H38/4443 are in different clusters, diverse from each other and easily distinguishable among the genotypes tested (Figure 1). A high degree of dissimilarity was also found between CB41-76 and CP52/68 (72.3%), CB41-76 and CO740 (71.3%) and CB41-76 and F134 (71.2%). This relationship clearly shows that these genotypes were geographically diverse and evolved independently. Genetic dissimilarity values ranging from 17-48% was reported by Selvi et al. (2005) using RFLP markers in tropical and subtropical Indian sugarcane cultivars. Chen et al. (2009) reported 56 to 91.6% similarity among sugarcane cultivars of different geographic origins with SSR markers. Genetic similarity coefficient ranging from 5.6 to 77.8% has been reported by Singh et al. (2010) using SSR in a collection of sugarcane species and commercial hybrids in India.

Both neighbour joining and PCoA plot based on pairwise genetic distances (Figures 1 and 2) assigned clones into three clear distinct clusters. Moreover, the clustering pattern at the lower level of hierarchies after the differentiation of the major clusters showed sub-grouping of the clones into 11 major groups. Starting from the bottom and moving clockwise in the dendrogram (Figure1) depicting groups with different colours, group I comprised 19 clones almost from every source country except India for Bihar Orissa (BO) varieties, Louisiana, Mauritius and Demerra X Barbados crosses from Barbados. Group II included clones N50/93, L60-14 and PR980 from South Africa (Natal), Louisiana and Puerto Rico respectively.

Group III contained clones H48/4605 from USA (Hawai) and C120-78 from Cuba. Group IV had two clones from Lousiana namely L60-25 and L60-35 and one clone E188/53 from unknown source. Group V had 41 clones from every source country except Cuba, Hawaii, Demerra and Formosa (Taiwan). Group VI consisted of four clones from Hawaii, India (Coimbatore), Formosa (Taiwan) and Barbados each contributing one clone. Group VII had two clones BO3 and M53/263 from Bihar Orissa (India) and Mauritius respectively. The next group (VIII) also had two varieties C105-73 and CO331 from Cuba and India (Coimbatore) respectively. Group IX had four clones CB38-22, D42/58, DB386/60 and E188/56 from Campos Brazil, Demerra, crosses from Demerra and Barbados

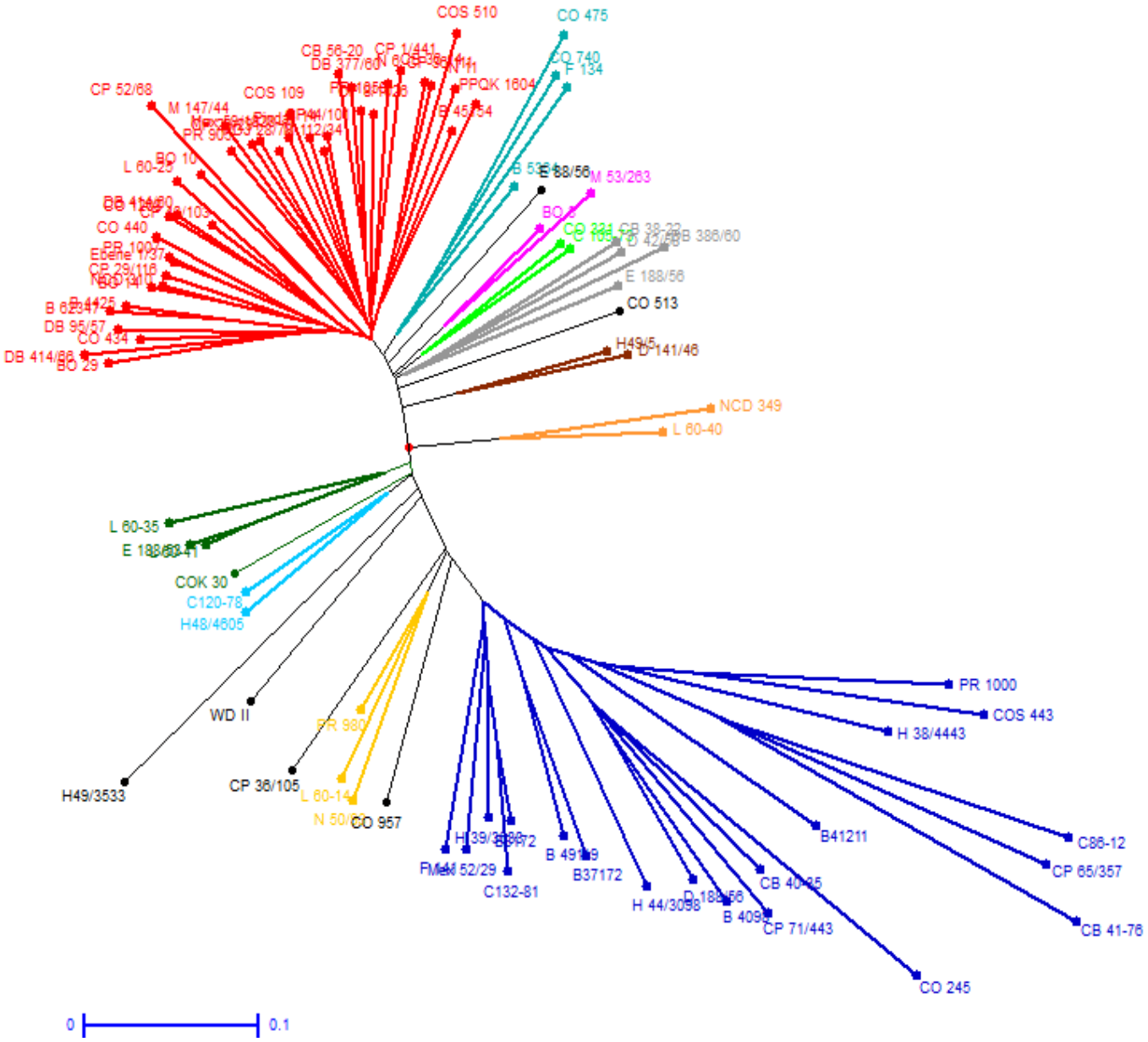

Figure 1. Unrooted UPGMA tree, calculated from SSR data (260 Loci), assembling 90 genotypes which were sugarcane species clones introduced into Ethiopia from different source countries around the world.

and unknown source. Group X consisted of two clones H49/5 (Hawaii) and D141/46 (Demerra). The last group XI contained two clones NCD349 and L60-40 from Barbados and Louisiana respectively.

Located between the different groups, seven clones namely CO957, CP36/105, WDII, H49/3533, COK30, E88/56 and CO513 did not cluster with any group. This relationship might be due to the reason that these genotypes were geographically diverse and evolved independently. Out of these seven clones COK30 and E88/56 are clones whose countries of origin were unknown. The other eight clones with unknown sources joined different clusters. Accordingly, COS443 found close to Puerto Rico variety PR1000 joined group I. E188/53 joined group IV with Louisiana varieties L60-25 and L60-35. Pindar, COS109, Ebene1/37, COS443 and COS510 clustered with accessions in group V. E188/56 joined group IX. The placement of these genotypes in the respective clusters might indicate their close similarity with the accessions with which they cluster in their evolutionary history.

Generally the results of the dendrogram and the PCoA analysis showed that regardless of their origin sugarcane cultivars from different source countries tend to cluster

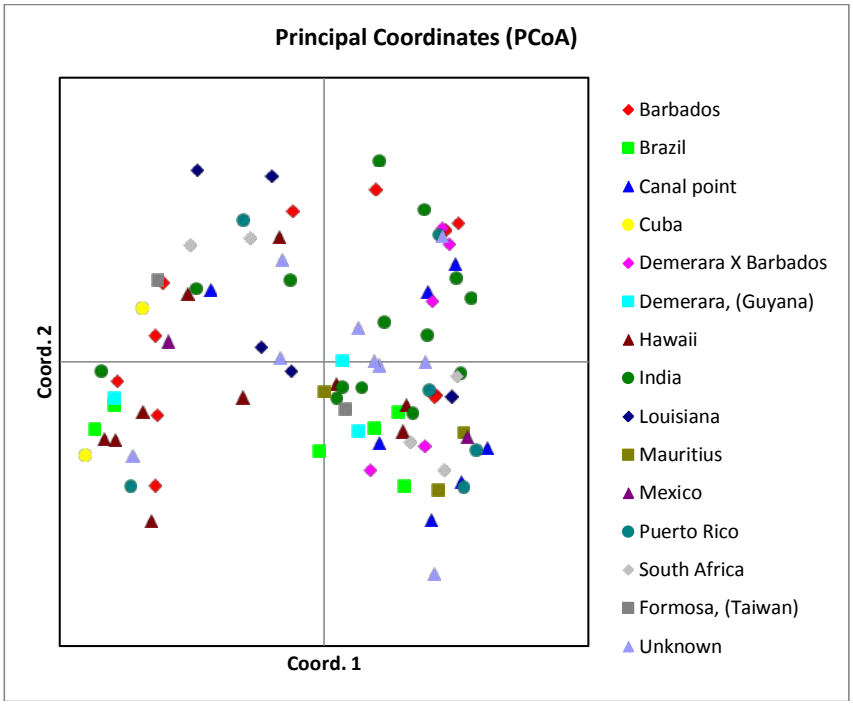

Figure 2. PCoA graph of 90 sugarcane accessions introduced into Ethiopia from 14 source countries and unknown sources around the world.

together and likewise accessions from the same country were often in different clusters (Figures 1 and 2) indicating the possibility of existence of shared alleles among the genotypes. Thus the present clustering pattern of clones also disclosed the possibility of close similarity between their evolutionary courses. No evidence was found for all genotypes with one geographical location to be single cluster in the clustering pattern of genetic diversity, thereby underscoring the importance of human-mediated gene flow in addition to artificial selection.

Principal coordinate analysis

The principal coordinate analysis (PCoA) allowed the separation of the genotypes into three gene pool groups regardless of the geographical area where the clones were introduced (Figure 2). Substantial diversity was found in the genotypes in the PCoA analysis and this was distributed both within and between gene pools. Similar to results of cluster analysis, accessions were seen clustering randomly regardless of country of origin

Results of the PCoA were in agreement with that of the neighbour-joining dendrogram, with three major clusters detected. Further differentiation into recognized clones belonging to each cluster was also observed as in the dendrogram. The overall variation explained by the PCoA was 74.96% with dimensions 1, 2 and 3 explaining 49.8, 13.69, and 11.47%, respectively.

Genetic diversity within and among accessions of country of sources

All of the microsatellite markers used in this study had highest number of polymorphic loci (Table 2). The proportions of polymorphic loci were high (86.54%) in accessions from India followed by 78.08 and 77.31% of the accessions from Barbados and Hawaii respectively (Table 5). Within populations number of different alleles was in the range of 0.523 in Cuba genotypes to 1.796 in the Indian accessions with a mean of 1.369. The average number of effective alleles per locus within the genotypes of the different countries of origin ranged from 1.144 in the genotypes of Cuba to 1.489 in those of Canal Point with a mean of 1.365 over loci and populations. Shannon's information index was highest (0.438) in Indian accessions followed by 0.411 in those of Canal Point with over all mean of 0.321 (Table 5). Gene diversity was highest (0.290) in accessions of India while the lowest (0.084) was recorded in those of Cuba with mean of 0.214 across populations (Table 5 and Figure 3). Generally, within populations, higher number of different alleles, higher number of effective alleles, high Shannon information index, higher level of gene diversity and the highest percentage of polymorphic loci were observed among Indian varieties (Table 5).

AMOVA results showed that 97% of allelic diversity was attributed to individuals within population while only 3% was distributed among populations (Table 4). This result may reflect the high degree of variation among

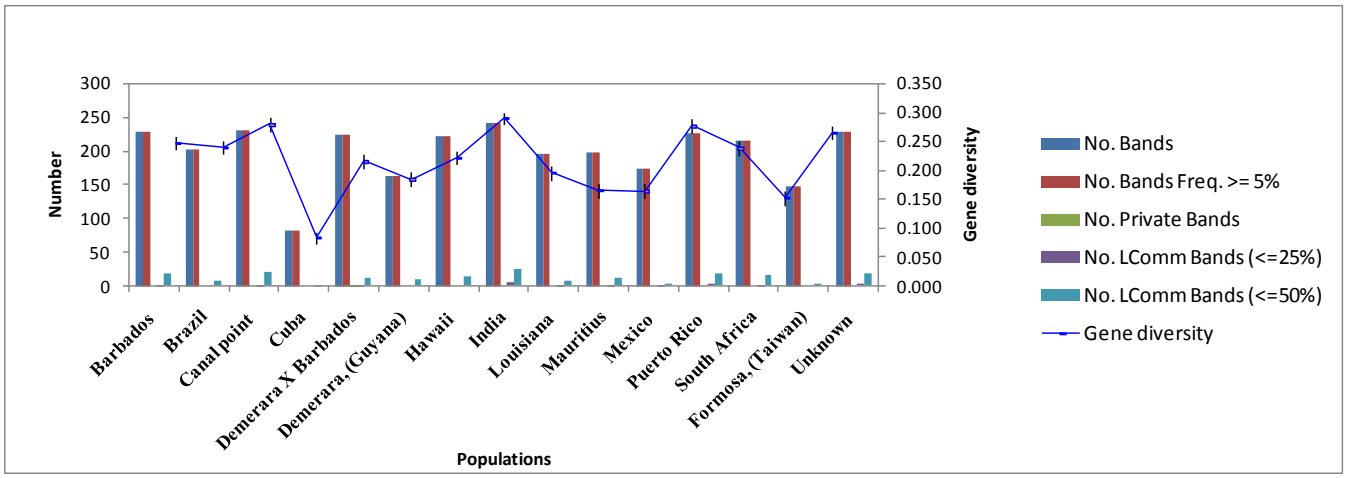

Figure 3. Band patterns across populations. No. LComm Bands<=25%)= No. of Locally Common Bands (Freq.>=5%) Found in 25% or Fewer Populations; No LComm Bands (<=50%)= No. of Locally Common Bands (Freq.>=5%) Found in 50% or Fewer Populations.

Table 4. Analysis of molecular variance (AMOVA) for 90 individual exotic sugarcane clones from different source countries based on the analysis of 260 SSR fragments generated using 22 primer pairs.

Source of variation	df	Sum of squares	Variance components	% of total variation
Among Populations	14	677.875	1.204	3
Within Populations	75	3102.880	41.372	97
Total	89	3780.756	42.576	100

Significance of comparisons; Hawaii vs Canal point, Hawaii vs Demerara X Barbados and Hawaii vs India P<0.01; Cuba vs Canal point, Demerara X Barbados vs Barbados, Demerara X Barbados vs Brazil, India vs Cuba, Louisiana vs Demerara X Barbados, Mauritius vs Demerara X Barbados, Mauritius vs Hawaii and Formosa (Taiwan) vs Demerara X Barbados P=0.05.

Table 5. SSR diversity for 22 microsatellite loci in 15 populations (genotypes from 15 respective source countries) of introduced sugarcane clones in Ethiopia.

Population	Na	Ne	I	GD	P%
Barbados	1.662	1.411	0.377	0.247	78.08
Brazil	1.481	1.402	0.361	0.239	70.00
Canal Point	1.627	1.489	0.411	0.279	74.23
Cuba	0.523	1.144	0.123	0.084	20.38
Demerara X Barbados	1.477	1.368	0.322	0.215	61.15
Demerara, (Guyana)	1.100	1.319	0.272	0.184	47.69
Hawaii	1.627	1.359	0.345	0.222	77.31
India	1.796	1.486	0.438	0.290	86.54
Louisiana	1.331	1.330	0.295	0.195	57.69
Mauritius	1.181	1.288	0.243	0.165	42.31
Mexico	1.062	1.280	0.240	0.164	39.62
Puerto Rico	1.619	1.481	0.409	0.276	74.62
South Africa	1.477	1.410	0.354	0.238	65.00
Formosa, (Taiwan)	0.935	1.261	0.223	0.153	36.92
Unknown	1.642	1.452	0.396	0.264	76.15
Mean	1.369	1.365	0.321	0.214	60.51
SE	0.013	0.006	0.005	0.015	4.94

N_a =number of different alleles; N_e=effective number of alleles; I=Shannon index; GD= genetic diversity according to Nei (1978) and %P=percentage of polymorphic loci No. LComm Bands (<=25%) = No. of Locally Common Bands (Freq. >= 5%) Found in 25% or Fewer Populations; No. LComm Bands (<=50%) = No. of Locally Common Bands (Freq. >= 5%) Found in 50% or Fewer Populations.

Table 6. Unbiased Nei's genetic distance (below diagonal) and genetic identity (above diagonal) among and between populations (genotypes from respective source countries), numbers representing populations here correspond to population numbers in Table 5.

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	***	0.981	0.953	0.939	0.887	0.978	0.978	0.977	0.965	0.862	0.937	0.971	0.974	0.950	0.962
2	0.020	***	0.956	0.950	0.887	0.973	0.984	0.973	0.955	0.871	0.932	0.966	0.962	0.969	0.961
3	0.048	0.045	***	0.856	0.958	0.947	0.932	0.977	0.946	0.935	0.939	0.982	0.982	0.920	0.978
4	0.063	0.051	0.155	***	0.778	0.945	0.967	0.901	0.903	0.756	0.869	0.902	0.892	0.904	0.904
5	0.120	0.120	0.043	0.251	***	0.896	0.856	0.939	0.890	0.891	0.889	0.932	0.931	0.867	0.926
6	0.022	0.027	0.055	0.057	0.109	***	0.988	0.968	0.964	0.873	0.927	0.979	0.965	0.940	0.980
7	0.022	0.016	0.071	0.034	0.155	0.012	***	0.956	0.952	0.838	0.914	0.973	0.955	0.939	0.968
8	0.023	0.027	0.023	0.105	0.062	0.033	0.045	***	0.967	0.909	0.938	0.973	0.972	0.956	0.967
9	0.036	0.046	0.056	0.103	0.116	0.036	0.049	0.033	***	0.882	0.926	0.967	0.976	0.950	0.966
10	0.149	0.139	0.068	0.279	0.115	0.136	0.177	0.095	0.126	***	0.895	0.910	0.909	0.853	0.900
11	0.065	0.070	0.063	0.140	0.117	0.076	0.090	0.064	0.077	0.111	***	0.932	0.942	0.942	0.926
12	0.030	0.034	0.018	0.103	0.071	0.021	0.027	0.027	0.033	0.094	0.071	***	0.982	0.934	0.992
13	0.027	0.039	0.019	0.114	0.071	0.036	0.046	0.028	0.024	0.096	0.060	0.018	***	0.934	0.975
14	0.052	0.032	0.083	0.101	0.143	0.062	0.063	0.045	0.051	0.159	0.060	0.068	0.068	***	0.924
15	0.039	0.040	0.022	0.100	0.077	0.020	0.032	0.033	0.035	0.105	0.077	0.008	0.025	0.079	***

sugarcane clones. Neil et al. (2009) studying on genetic diversity among main land USA sugarcane cultivars by SSR genotyping also reported molecular variation of 3.4% between populations and 96.6% within populations.

Among accessions of different source countries highly significant variation for molecular diversity ($P<0.01$) was observed between populations of Hawaii vs Canal point, Hawaii vs Demerara X Barbados and Hawaii vs India while the variation was significant ($P<0.05$) between Cuba vs Canal point, Demerara X Barbados vs Barbados, Demerara X Barbados vs Brazil, India vs Cuba, Louisiana vs Demerara X Barbados, Mauritius vs Demerara X Barbados, Mauritius vs Hawaii and Formosa (Taiwan) vs Demerara X Barbados. The molecular variation was not significant between other pairs of populations denoting shared alleles among them. This was supported by the results of the dendrogram in which identities of cultivars with respect to country of sources did not contribute to the clustering pattern because individual clusters include cultivars form different countries. This suggests that some genetically similar cultivars may be present in the different countries. In the dendrogram, it was also observed that accessions of countries with high molecular variation mostly didn't cluster together. Future breeding efforts involving crosses between accessions of countries with high molecular variation may provide beneficial genes and alleles in new sugarcane varieties while maintaining genetic diversity.

Population differentiation

Analysis of genetic differentiation among the accessions of countries revealed F_{ST} values ranging from 0.0024 (between Demerara (Guyana) and South Africa) to 0.5134 (between Cuba and India), which was a very large differentiation with an overall average of 0.059 (Table 7). High differentiation ($F_{ST}=0.4113$, $p<0.05$) was also observed between Barbados and Cuba and Canal Point and Cuba ($F_{ST}=0.4112$, $p<0.05$). Moderate gene differentiation among many pairs of populations was also evident (Table 7). The average gene flow (Nm) among populations was 1.7213. Nei's unbiased genetic distance ranged from 0.018 (between accessions of Puerto Rico and South Africa) to 0.279 (between accessions of Cuba and Mauritius) (Table 6) with an overall average of 0.053. Genetic identity values were in the range of 0.756 to 0.992 with overall average of 0.950 (Table 6). From these results, we can therefore say that a high percentage of genetic diversity is distributed among populations. This can give a clue that some exotic genotypes have their unique genetic

Table 7. Pair-wise F_{ST} values of the 15 populations (genotypes from respective source countries) of introduced sugarcane clones in Ethiopia, numbers representing populations here correspond to population numbers in Table 5.

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.0000														
2	0.0128	0.0000													
3	0.0567	0.0526	0.0000												
4	0.4113	0.2523	0.4112	0.0000											
5	0.0988	0.0911	0.0160	0.3911	0.0000										
6	0.0716	-0.0047	0.0860	0.1104	0.0912	0.0000									
7	0.0136	-0.0041	0.1133	0.3408	0.1658	0.0218	0.0000								
8	0.0244	0.0508	0.0213	0.5134	0.0556	0.1537	0.0851	0.0000							
9	0.0439	0.0341	0.0872	0.2586	0.1013	0.0031	0.0570	0.0858	0.0000						
10	0.1701	0.0977	0.0467	0.3469	0.0869	0.0462	0.1821	0.1686	0.0890	0.0000					
11	0.1043	0.0235	0.0490	0.1237	0.0414	-0.0732	0.1013	0.1934	0.0245	-0.0296	0.0000				
12	0.0137	0.0175	-0.0192	0.3407	0.0245	0.0424	0.0391	-0.0032	0.0217	0.0480	0.0498	0.0000			
13	0.0277	0.0112	-0.0062	0.2991	0.0088	0.0024	0.0527	0.0330	0.0143	0.0448	0.0133	-0.0274	0.0000		
14	0.1090	0.0102	0.1312	0.0231	0.1092	-0.0617	0.0871	0.2095	-0.0044	0.0732	-0.0689	0.0827	0.0381	0.0000	
15	0.0288	0.0308	0.0147	0.4076	0.0603	0.0560	0.0490	0.0286	0.0484	0.1058	0.0971	-0.0192	0.0124	0.1327	0.0000

constitution apart from each other which can be exploited for improvement of the crop in the future sugarcane breeding program of the country.

Conclusion

The present genetic analysis based on the segregation at SSR loci provides genetic information on the introduced sugarcane genotypes in the country. The genetic relationship information of the cultivars will help sugarcane breeders to select the appropriate parents in their breeding programs to maximize yield as well as to maintain genetic diversity.

ACKNOWLEDGEMENT

The authors are grateful to the financial grant of the Sugar Corporation of Ethiopia. Sugarcane Breeding Institute, Tamil Nadu State, Coinbatore, India is duly acknowledged for hosting the laboratory work.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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Vol. 13(46), pp. 4320-4329, 12 November, 2014
DOI: 10.5897/AJB11.079
Article Number: CCF834A48569
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Evaluation of some handling and processing parameters for briquetting of guinea corn (*Sorghum bi-color*) residue

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Received 1 July, 2011; Accepted 25 April, 2012

Biomass materials require reduction and densification for the purpose of handling and space requirements. Guinea corn (*Sorghum bi-color*) is a major source of biomass material in the tropic regions. The densification process involves some measurable parameters, namely: pressure, particles size and binder ratio. Guinea corn residue was collected from the Teaching and Research Farm of the Federal Polytechnic at Bida in Nigeria. The moisture content was 9.08% dry basis (db). It was reduced and sieved into three particle sizes D_1 (4.70 mm), D_2 (1.70 mm) and D_3 (0.60 mm). Starch paste of 40, 45, 50 and 55% was added as binder. Briquettes were produced using a hydraulic press and a cylindrical die (56 mmϕ) at the processing pressures of 7.5, 8.5, 9.5 and 10.5 Megapascal (MPa). The bulk density of the unprocessed material was 46.03 kg/m^3. The mean relaxed briquettes bulk density was 208.15 kg/m^3, which reflects a volume reduction of about 450%. The maximum density of the briquettes ranged from 789 to 1372 kg/m^3. For the expansion characteristics, the maximum and minimum axial relaxation occurred in the first 30 min of the extrusion. All the processing parameters were found to be significant at $P<0.05$ test level for all the measured characteristics. The briquettes were kept for six months under ambient condition without deterioration.

Key words: Guinea corn, briquettes, residue, parameters.

INTRODUCTION

Mechanics of agricultural material as a scientific discipline is presently being developed. For now, there are many process-material interactions that do not have exact methods of representation. Nevertheless, the experimental methods developed so far, can somehow be used successfully to select, design and optimize machines, (Sitkei. 1986). Heinimo (2008) postulated that at present, biomass covers approximately 11% of the global total primary energy consumption of slightly more than 430 EJ/year. Briquetting of forest products, agricultural wastes and rural-agro industrial residues has long been recognized as a viable technology for

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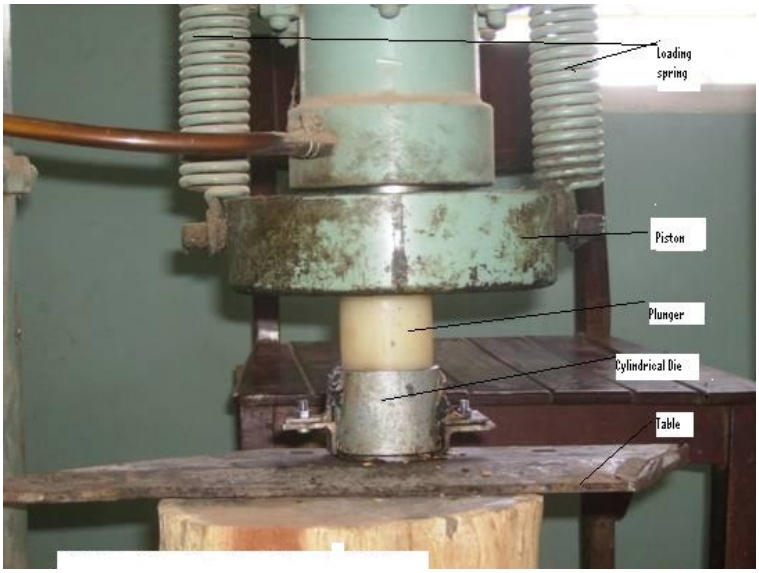

Figure 1. Hydraulic powered press fitted with plunger and cylinder die.

alternative energy generation (Resch, 1982). This statement is also buttressed by Gurkan et al. (2010). Briquetting technology is fast gaining prominence in the world of science today because of its numerous advantages of helping to densify very bulky (low density) materials, particularly those of biomass origin.

The compaction of agricultural residues into briquettes in general terms improves the economics of material handling, transportation and storage, thereby enhancing more versatile application in use. Munoz-Hernandez et al. (2004), identified some methods of achieving densification using commercial machines which include bailing, cubing, pelleting and briquetting; by means of piston, extrusion screws or by roll presses. Furthermore, it was identified that one of the requirements to design, construct and improve densification system is based mainly on the knowledge of suitable levels of process variables such as die geometry, relaxation time, die and material temperature and pressure. Other material variables include moisture content and its distribution, size and shape of the particles, size distribution of the particles, biochemical and mechanical characteristic (Rehkugler and Buchele, 1969). Although the effect of binder is still under intense research, Tabil et al. (1997) concluded that, there exists little scientific information related to the effectiveness of binders. However, a few researchers have postulated that at relatively low pressure application, the effect of binder might enhance densification. Starch has been used as a binding agent for briquetting of Rattan furniture waste and guinea corn (*Sorghum bi-color*) residue (Olorunshola, 2004; Bamgboye and Bolufawi, 2009).

Some researchers have used mechanical elements of commercial machines as experimental prototypes for densification, but the constraints here include, cost of instrumentation and large volumes of materials required for the experiment. Experimental tests by means of presses and laboratory dies for densification have offered easier and less expensive ways to conduct experimental tests under controlled conditions. Under laboratory conditions, technical information needed for economic and technical decisions can be obtained; for instance, in a closed-end die, the temperature and the use of binder can be controlled with high precision such as achieved by Faborode and O'Callaghan (1987) and Mohsenin and Zaske (1976).

The main objectives of this work was to find the levels of some selected processing parameters that provide optimum responses in terms of some measured post briquetting characteristics in briquetting of guinea corn residue. The parameters selected include pressure, particle size distribution, binder quantity and dwell-time.

MATERIALS AND METHODS

A 3 x 4 x 4 randomized complete block design (RCBD) experiment was used to synchronize the parameters of pressure (P), particle size (D) and binder/residue ratio (B) (Gomez and Gomez, 1983) adopting a dwell-time of 90 s. The operating pressures were 7.5, 8.5, 9.5 and 10.5 Megapascal (MPa). The particle size distribution was obtained by size reduction (milling) and sieve analysis. The particle sizes obtained were distributed into three categories: D_1 = 4.70 mm, D_2 = 1.70 mm and D_3 = 0.60mm. The binder/residue ratio was 40, 45, 50 and 55% by weight of binder/residue. Each sample briquette was produced using a fixed die charge packed in a steel cylinder 56 mm diameter positioned and compressed in a hydraulic press as shown in Figure 1. The briquettes formed are displayed in Figure 2.

Some measured characteristics of the briquettes included the bulk and initial densities, maximum and relaxed densities and the rate of expansion. The compaction energies were also determined.

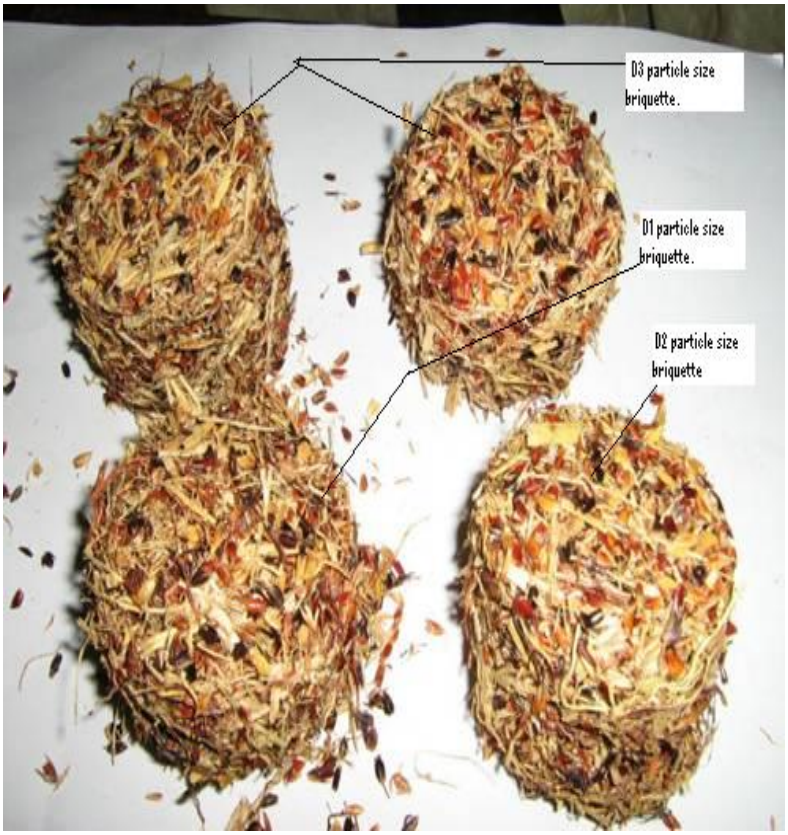

Figure 2. Briquettes produced form the hydraulic press.

Table 1. Initial density (Y_o) Kg/m^3.

Binder ratio (%)	Particle size D_1 (4.7 mm)	Particle size D_2 (1.7 mm)	Particle size D_3 (0.6 mm)
B_1	141	208	175
B_2	149	212	205
B_3	146	216	246
B_4	140	247	250

P, Pressure in Megapascal (MPa); D, particle size in mm; B, binder ratio; Y, density kg/m^3; r, Y_d/Y_o; r_d, Y_r/Y_d; r_r, $1/r_d$.

Other characteristics determined included compaction ratio (r) and relaxation ratio (r_r) which are derivatives of the initial, maximum and relaxed densities. The readings were treated statistically using, means and analysis of variance (ANOVA). Correlation and regression analysis were used to streamline the statistically treated data. The various densities and expansion rates were subjected to ANOVA test to determine the level of significance of the processing parameters (P, D and B) for the measured characteristics. The shelf live was estimated after observing the briquette for the period of six months under ambient condition.

RESULTS AND DISCUSSION

The various densities are in kg/m^3: initial density (Y_o) (zero pressure application), maximum density (Y_d) (in-die density) and relaxed density (Y_r) are displayed in Tables 1, 2 (a, b and c) and 3 (a, b and c).

A careful study of the data in Tables 1 to 3 reveal the following observations: the maximum densities for the particle size D_1, D_2 and D_3 varied from 798 to 1372 kg/m^3 as shown in Tables 2a, b and c. These values are much higher than the initial densities of the uncompressed mixture (141 to 250 kg/m^3) shown in Table 1. Thus, this process has been able to obtain improved density which is a desirable factor in briquetting. The bulk density of the unprocessed material was 46.03 kg/m^3 while the mean bulk density of the relaxed briquette was 208.15 kg/m^3, reflecting a volume reduction of about 450%. An increase in the maximum density was observed for all the particle

Table 2a. Maximum density (Y_d) Kg/m^3 (D_1).

Binder ratio (%)	P_1	P_2	P_3	P_4
B_1	1161	1204	1292	1372
B_2	1018	1074	1192	1307
B_3	899	924	987	1050
B_4	789	888	917	1015

P, Pressure in Megapascal (MPa); D, particle size in mm; B, binder ratio; Y, density kg/m^3; r, Y_d/Y_o; r_d, Y_r/Y_d; r_r, $1/r_d$.

Table 2b. Maximum density (Y_d) Kg/m^3 (D_2).

Binder ratio (%)	P_1	P_2	P_3	P_4
B_1	917	980	1030	1110
B_2	899	960	996	1093
B_3	892	932	994	1053
B_4	872	911	932	938

P, Pressure in Megapascal (MPa); D, particle size in mm; B, binder ratio; Y, density kg/m^3; r, Y_d/Y_o; r_d, Y_r/Y_d; r_r, $1/r_d$.

Table 2c. Maximum density (Y_d) Kg/m^3 (D_3).

Binder ratio (%)	P_1	P_2	P_3	P_4
B_1	821	960	1015	1093
B_2	789	928	960	980
B_3	789	861	917	960

P, Pressure in Megapascal (MPa); D, particle size in mm; B, binder ratio; Y, density kg/m^3; r, Y_d/Y_o; r_d, Y_r/Y_d; r_r, $1/r_d$.

Table 3a. Relaxed density (Y_r) Kg/m^3 (D_1).

Binder ratio (%)	P_1	P_2	P_3	P_4
B_1	235	242	244	248
B_2	238	240	244	235
B_3	239	240	240	238
B_4	281	304	289	292

P, Pressure in Megapascal (MPa); D, particle size in mm; B, binder ratio; Y, density kg/m^3; r, Y_d/Y_o; r_d, Y_r/Y_d; r_r, $1/r_d$.

Table 3b. Relaxed density (Y_r) Kg/m^3 (D_2).

Binder ratio (%)	P_1	P_2	P_3	P_4
B_1	347	357	352	248
B_2	334	334	328	245
B_3	329	330	325	243
B_4	324	323	323	292

P, Pressure in Megapascal (MPa); D, particle size in mm; B, binder ratio; Y, density kg/m^3; r, Y_d/Y_o; r_d, Y_r/Y_d; r_r, $1/r_d$.

Table 3c. Relaxed density Kg/m^3 (D_3).

Binder ratio (%)	P_1	P_2	P_3	P_4
B_1	389	405	415	436
B_2	427	435	424	433
B_3	388	400	397	406

P, Pressure in Megapascal (MPa); D, particle size in mm; B, binder ratio; Y, density kg/m^3; r, Y_d/Y_o; r_d, Y_r/Y_d; r_r, $1/r_d$.

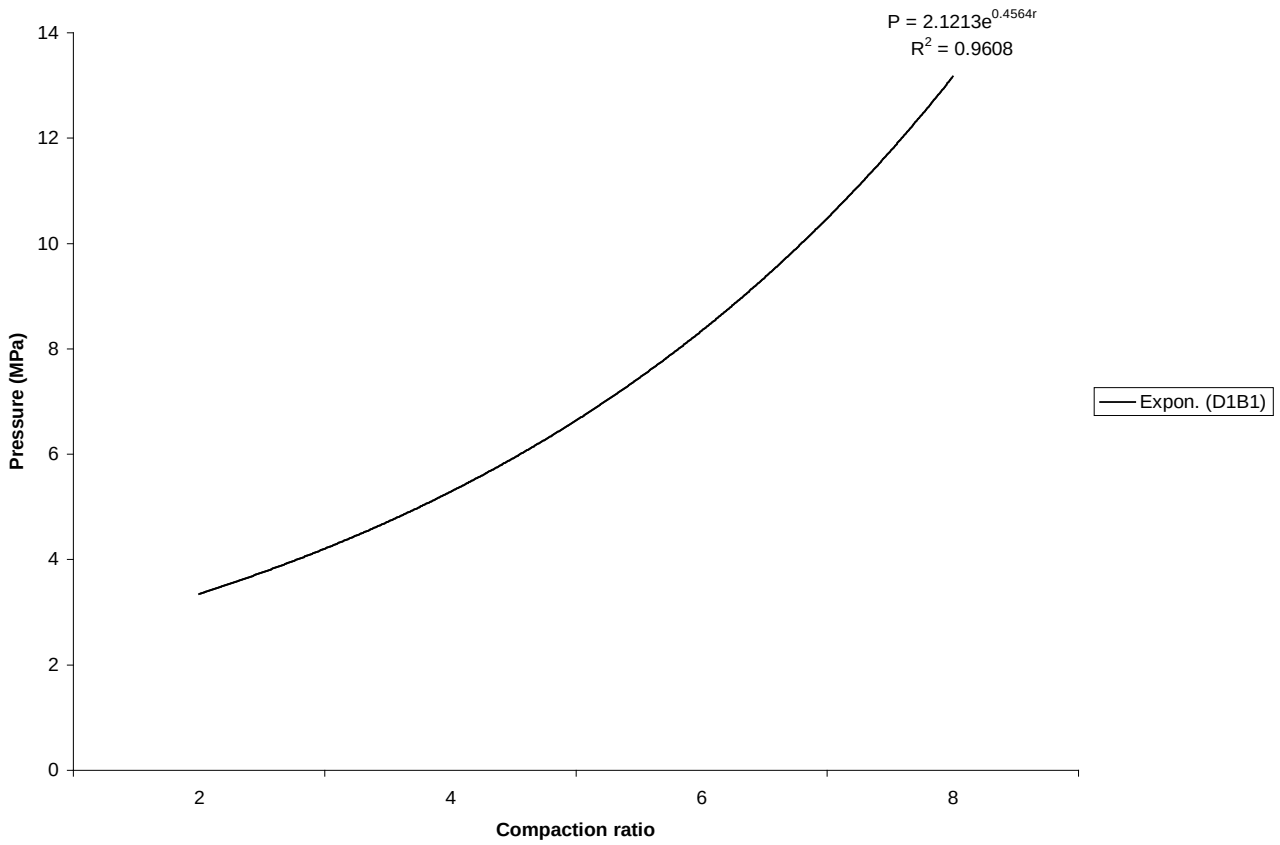

Figure 3. Relationship between pressure and compression ratio for D_1B_1.

sizes with increasing pressure. A decrease in density was also observed with increasing binder ratio. The relaxed briquette densities shown in Tables 3a, b and c were smaller than the recorded maximum densities because of the expansion of the briquette after extrusion.

The ANOVA showed that the effect of the processing parameters, pressure, binder ratio and particle size were significant at $P < 0.05$. The relationship between the selected parameters and some measured characteristics are as shown in Figures 3 to 10. The effects of pressure and compaction ratio on the particle sizes of the material are graphically shown in Figures 3 to 5. The graphs are exponential in nature. The area under each curve in Figures 3 to 5 are estimates of the compaction energy. The energies of compaction are found to reduce with reducing particle size dimension. The rates of expansion of the briquettes considering the binder ratio at the background are illustrated by Figures 6 and 7. It was observed that the most rapid rate of expansion took place in the first 100 min after extrusion. Figures 8 to 10 showed the effect of binder on maximum and relaxed density for briquettes with D_1, D_2 and D_3 particle size. For the expansion characteristic (axial relaxation), the maximum and minimum axial relaxation occurred in the first 30 min of the extrusion with values 138.64 and 28.0% in the longitudinal axis while the maximum and minimum radial relaxation were 11.50 and 4.0%, respectively. For briquettes with D_1 particle size, the maximum density decreases with increasing binder ratio indicating that more of the spaces have been filled with binder materials which to some extent is not compressible. The relaxed density does not exhibit the phenomenon

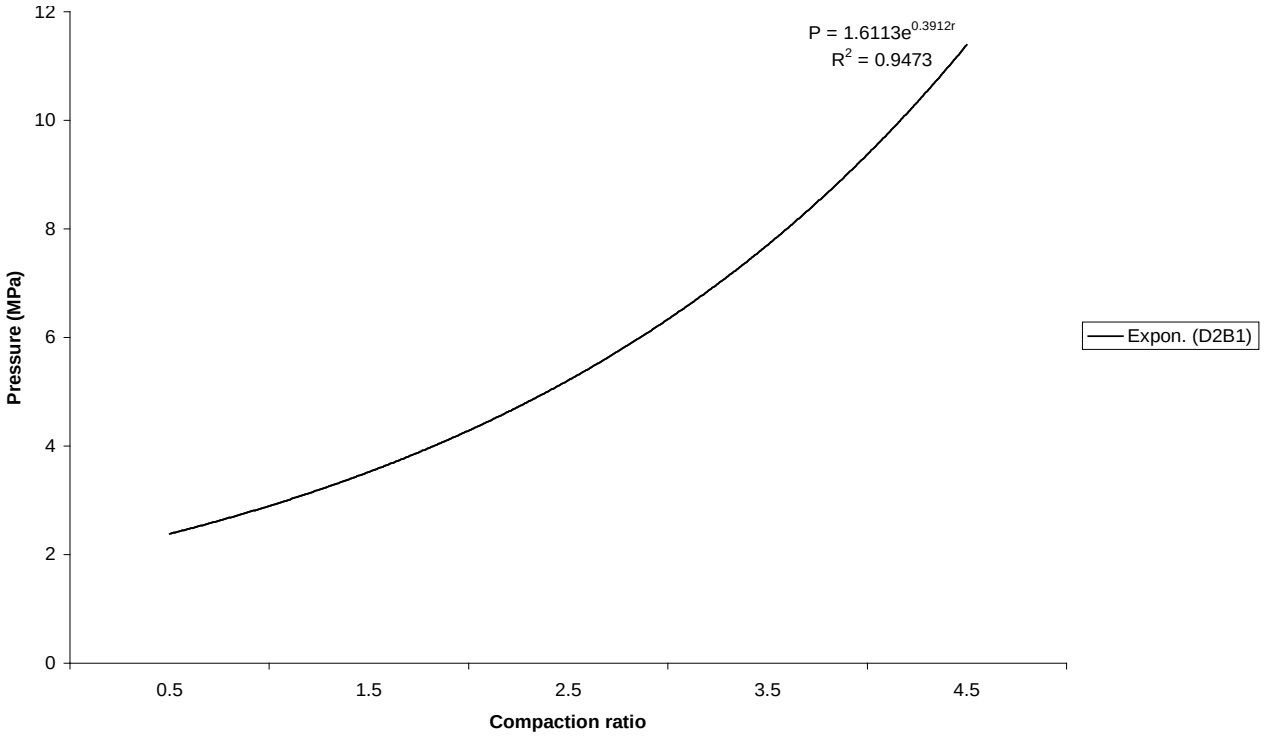

Figure 4. Relationship between pressure and compression ratio for D_2B_1.

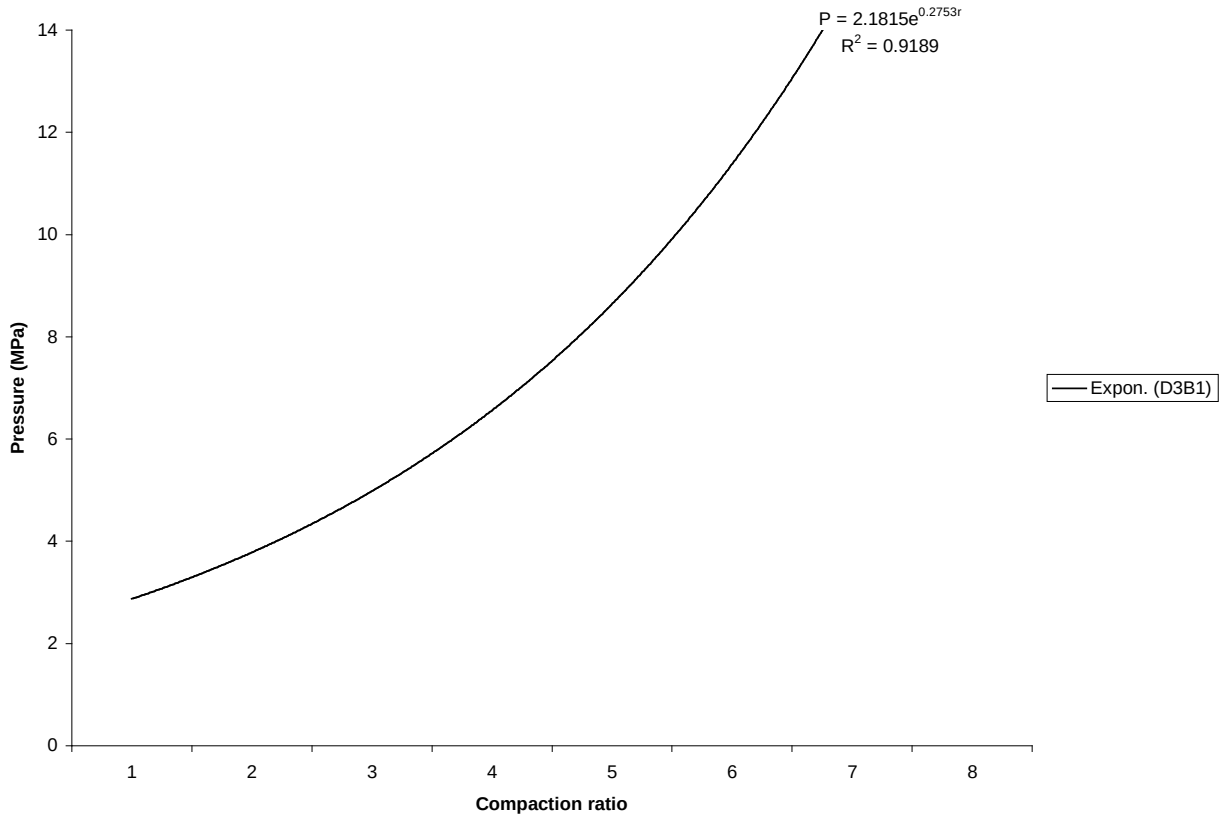

Figure 5. Relationship between pressure and compression ratio for D_3B_1.

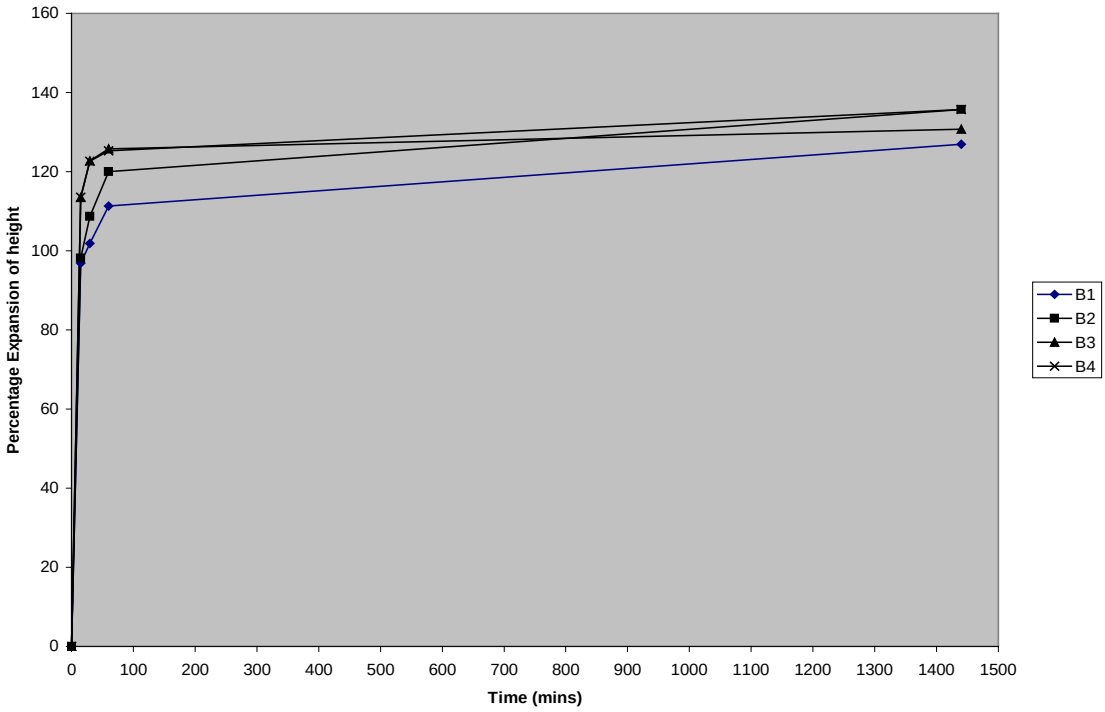

Figure 6. Percentage rate of expansion of briquettes with binder ratio (D_2P_1).

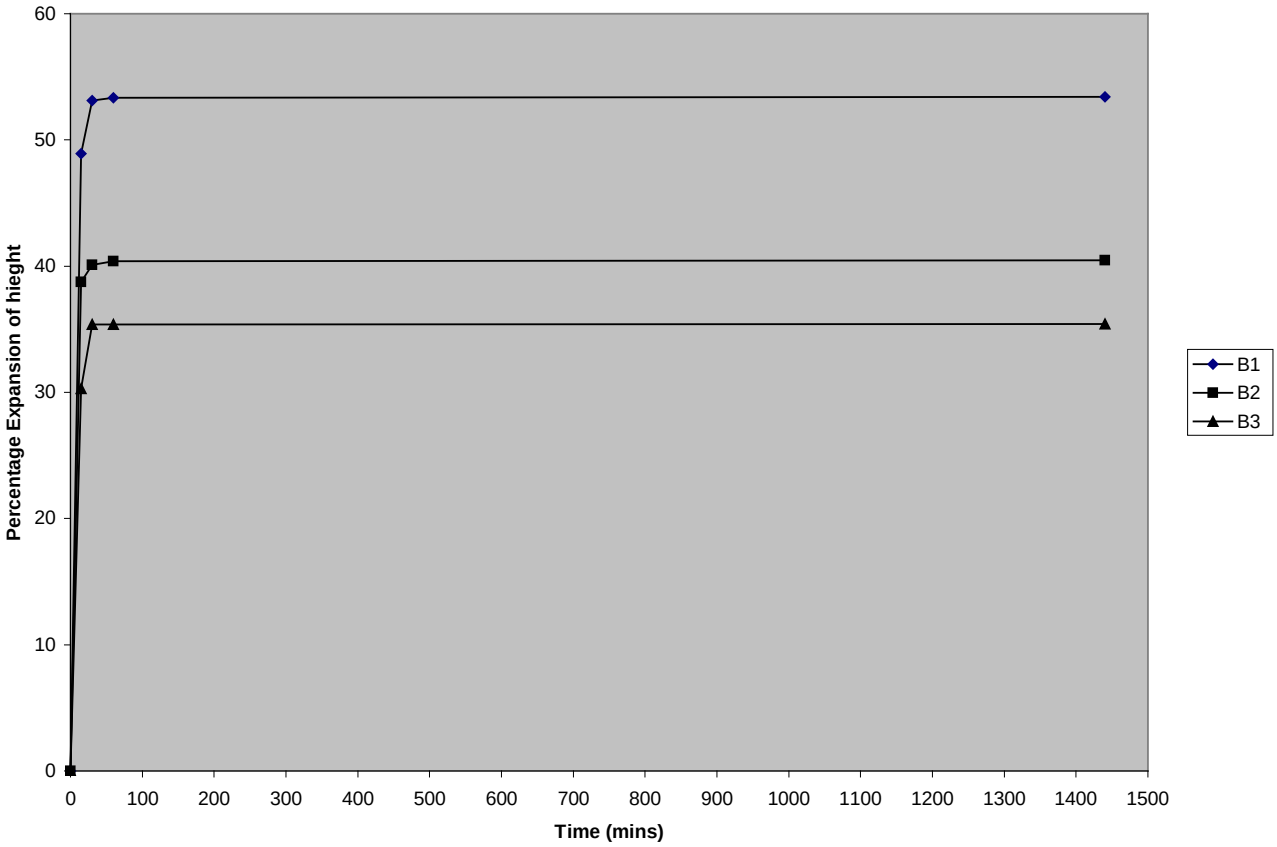

Figure 7. Percentage rate of expansion of briquettes with binder ratio (D_3P_1).

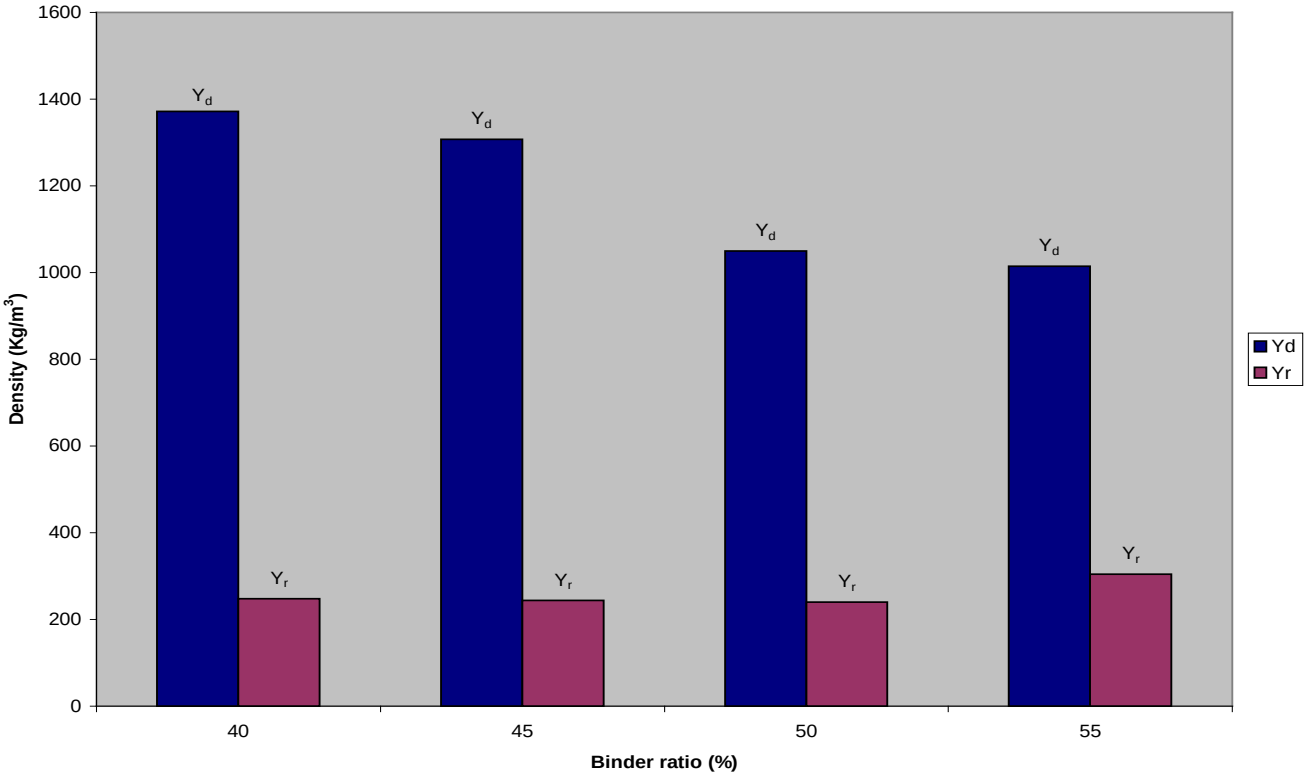

Figure 8. Effect of binder ratio on maximum and relaxed density for D_1 particle size briquettes.

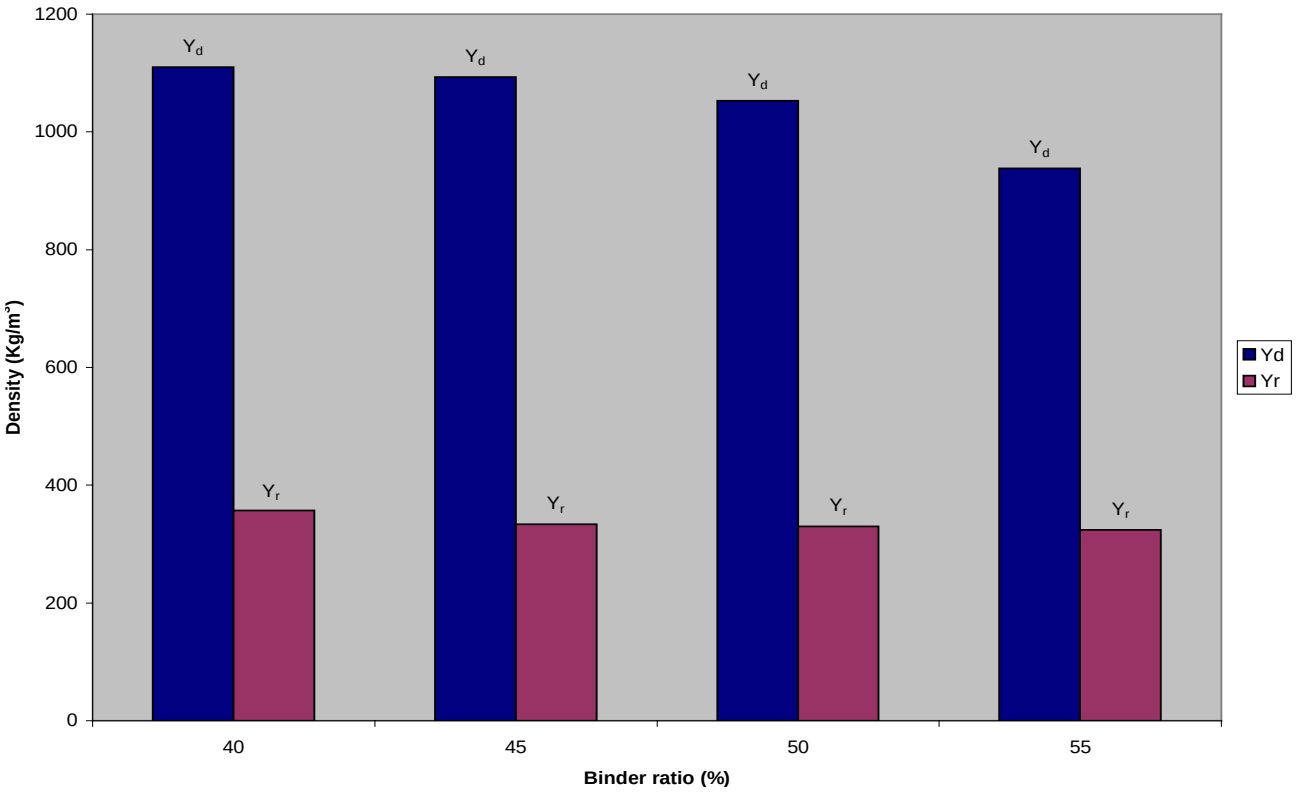

Figure 9. Effect of binder ratio on maximum and relaxed density for D_2 particle size briquettes.

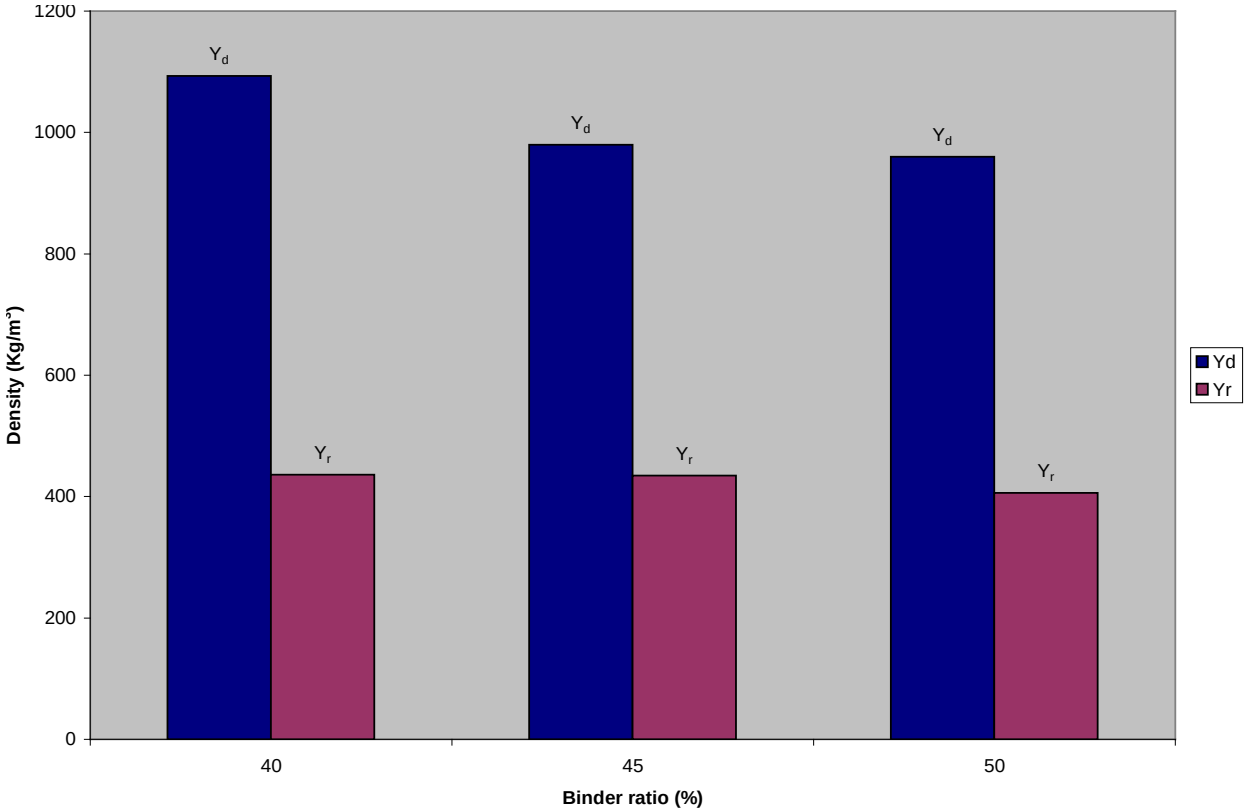

Figure 10. Effect of binder ratio on maximum and relaxed density for D_3 particle size briquettes.

raised above. For D_2 particle size briquettes, there is very little variation in the maximum and relaxed densities.

This phenomenon is traceable to the lustre surface nature of the particle size. For the briquettes with D_3 particle size, the trend was similar to what was observed for D_2 particle size briquettes even though the particles were finer. The briquettes that were kept for shelf life examination did not disintegrate appreciably over a period of about six months, when visually inspected and physically handled.

Conclusion

The measured characteristics indicated that each and every one of the processing parameters of pressure, particle size and binder ratio have singularly or corporately affected the values of the measured characteristics. The compaction energy was directly related to the particle size more than the other processing parameters of pressure and binder ratio. Less compaction energy was recorded for smaller particle size compression process. A large volume reduction (450%) of the material was achievable by these processes. All the processing parameters were found to be significant at $p < 0.05$ for all the measured characteristics. If the right combination of the handling and processing parameters are applied to the materials, the briquettes can be sustained for a long period. For further work, energy balance for size reduction and compression process could be determined by investigation.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Vol. 13(46), pp. 4330-4337, 12 November, 2014
DOI: 10.5897/AJB2014.13868
Article Number: 6017F0D48571
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effect of equation pro and kema zed fungicides on cellulase and pectinase enzymes produced by some phytopathogenic fungi of broad bean

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Received 17 April, 2014; Accepted 16 May, 2014

Two fungicides (equation pro and kema zed) were added to the medium in five doses (50,100, 200, 300 and 400 ppm active ingredient) to investigate the chemical control of cellulase and pectinase enzymes produced by some plant pathogens of broad bean. *Alternaria alternata*, *Alternaria citri*, *Alternaria* and *Cochliobolus spicifer* were isolated from diseased leaves of broad bean. The incorporation of these fungicides into the media for cellulase and pectinase enzymes exhibited an inhibitive effect on both cellulase and pectinase enzymes by all doses used except in few cases; the production of exo- and endo-β-1, 4-glucanase enzymes were slightly increased compared to the control at 50 and 100 ppm doses. The inhibitive effect of equation pro on endo- and exo-β-1,4-glucanase production ranged between 5.9 to 70.4% by *A. alternata*, *A. citri* and *C. spicifer*. However, kema zed exhibited an inhibitive effect on cellulases which ranged between 1.9 to 51.9%. On the other hand, the inhibitive effect of Equation pro on pectinase enzyme ranged between 20.1 to 75.6% by *A. citri* and *A. raphani* while kema zed exhibited an inhibitive effect ranged between 13.5 to 62.8%. The inhibitive effect of these fungicides on the mycelial growth of tested fungi was nearly similar to those for the enzyme production.

Key words: Fungicides, cellulase and pectinase enzymes, broad bean fungi.

INTRODUCTION

All enzymes consist of protein having catalytic properties and a non-protein part called a prosthetic group or coenzyme. Pesticides can interact either with the protein part of the enzyme molecule and completely inactivate it, or with the coenzyme and form stable compounds or unstable complexes. In both cases, the pesticides are classified as enzyme inhibitors (Gruzdyev et al., 1983).

The effect of fungicide was quite variable with different enzymes and fungal species. The biosynthesis of various enzymes was found to be controlled by a number of fungicides (Omar and Abd-Alla, 2000; Moharram et al., 2004; 2011; Gopinath et al., 2006; Dornez et al., 2008;

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Özer et al., 2010; Saleem et al., 2012). There are several reports indicating the varying effect of fungicides on both mycelial growth, cellulase and pectinase production according to different factors including the kinds and doses of fungicides and the fungal species tested (Arinze and Yubedee, 2000; Moharram et al., 2004; Rathod and Chavan, 2010; Saleem et al., 2012). Gopinath et al. (2006) studied the effect of Propiconazole, Difenoconazole and Carbendazim fungicides on growth and hydrolytic enzymes production by *Colletotrichum capsici*. Three fungicides inhibited mycelial growth (radial growth and mycelial biomass) of *C*. *capsici* compared to controls. Among the fungicides, Propiconazole exhibited the highest level of inhibition followed by Difenoconazole and Carbendazim. The incorporation of fungicides into the growth medium, significantly reduced production of polygalacturonase, polygalacturonase trans-eliminase, pectin trans-eliminase and cellulases by *C*. *capsici*. The highest degree of inhibition was observed in Propiconazole, followed by Difenoconazole and Carbendazim.

Recently, Özer et al. (2010) evaluated the influence of nine fungicides on mycelial growth and poly-galacturonase activity of *Botrytis cinerea*. All fungicides except Triadimenol and Tebuconazole inhibited mycelial growth of fungal isolates. Cyprodinil + Fludioxonil, Myclobutanil and Imazalil inhibited polygalacturonase activity more than 50%. Fenhexamid had a lower inhibitory effect (<50%) on polygalacturonase activity. Procymidone and Pyrimethanil induced both PG activity and isoenzyme banding profile of isolates sensitive to these fungicides.

Hydrolytic enzymes play an important role in the pathogenicity of plants by facilitating fungal penetration through the host cell wall (Wanjiru et al., 2002). The major components of plant cell walls are cellulose, hemicellulose and lignin, with cellulose being the most abundant component (Han et al., 2003). Cellulose consists mainly of long polymers of β 1-4, linked glucose units and forms a crystalline structure (Shallom and Shoham, 2003). Cellulase enzymes complex is a multi-domain protein that consists of three major enzymes components which are endo-β-(1-4)-D-glucanase, exo-β-(1-4)-D-glucanase and β-glucosidase that works syner-gically in complex cellulose degradation (Duff et al., 1987). Generally, fungi produce three major types of cellulolytic enzymes: endoglucanase, exoglucanase and cellobiohydrolase (Klyosov, 1990). These enzymes are extracellular and inductive in nature (Enari, 1983). The ability to produce cellulase are widespread among fungi and has become the subject of extensive investigation (El-Said, 2001; Moharram et al., 2004; El-Said et al., 2005; Narasimha et al., 2006; Shanmugam et al., 2008; Abu-Bakar et al., 2010; Gautam et al., 2010; Sherief et al., 2010; Sarkar et al., 2011). Pectins are high molecular weight polysaccharides found in higher plants. They form the primary cell wall and the main components of the middle lamella (Alkorta et al., 1998). The hydrolysis of pectin backbone is obtained by the synergistic action of several enzymes (Gummadi and Panda, 2003). Among the enzymes secreted, polygalacturanase and pectinase are responsible for cell maceration and death of plant tissue (Fernando et al., 2001).

The involvement of pectic enzymes in the degradation of pectic constituents of cell walls and middle lamella of plant tissues has been reported for diverse types of diseases (Ramos et al., 2010). Enzymes that attack pectic substances in the plant cell wall play a major role in pathogenicity (Cole et al., 1998). The role of pectin degrading enzymes in causing cell wall degradation is so important that it determines the virulence of many pathogens (Rogers et al., 2000). This investigation was carried out to study the effect of two fungicides commonly used in Egypt against phytopathogenic fungi of broad bean specially cell wall degrading enzymes which contribute in the first step of pathogenicity by penetrating of cell wall.

MATERIALS AND METHODS

Isolation of fungi

Fungi were isolated from 50 infected leaves samples of broad bean (*Vicia faba* L.) collected from different fields in Qena, Upper Egypt.

Effect of fungicides on cellulase enzymes

Equation pro and Kema zed fungicides were investigated for their effect on cellulaes and pectinase enzymes by the most active fungi for enzyme production. The chemical names, structural formula, active ingredient, manufacturers and agricultural uses of fungicides are shown in Table 1. Fungal isolates including *A. citri* and *Cochliobolus spicifer* (for exo-β-1,4-glucanase, C_1) and *A. alternata* and *A. citri* (for endo-β-1,4-glucanase, C_x) were cultured on 50 ml of the liquid medium containing cellulose or Carboxy methyl cellulose (CMC) as substrates. pH was adjusted and 6.50 ml of media were dispensed into each 250 ml Erlenmeyer conical flasks which were autoclaved for 15 min at 1.5 atm. Different doses of fungicides (50, 100, 200, 300 and 400 ppm, active ingredient) were individually added to the sterilized liquid medium under aseptic conditions. Media without fungicide served as control. Each flask was inoculated with two agar mycelial discs (10 mm diameter) of the tested fungal isolates obtained from a 7 days old culture. Inoculated flasks were incubated for 6 days at 30°C. Cultures were filtered and the filtrates were centrifuged at 4°C for 15 min at 15000 rpm. The clear supernatants were assayed for cellulase activity. Mycelial dry weights of the tested fungi were also determined.

Assay for cellulase activity (C_1 and C_x enzymes)

The method described by Nelson (1944) and modified by Naguib (1964) was employed as follows: 1 ml of each 1% cellulose powder for C_1-cellulase or CMC for C_X-cellulase was added separately to 1 ml of acetate buffer (pH = 6) and 1 ml of each culture filtrate. The

Table 1. Fungicides used their chemical names, active ingredients, manufacturers and agricultural uses.

Trade name	Equation Pro 52.5% WG	Kema zed 50%
Chemical name	Famoxadone: 3-anilino-5-methyl-5-(4 phenoxyphenyl)-2,4-oxazolidinedione. Cymoxanil: 2-2(2-cyano-2-methoxy iminoacetyl)-3-ethylurea.	Methylbenzimidazol-2-ylcarbamate
Active ingredient	Famoxadone 22.5% and Cymoxanil 30%	Carbendazim 50%
Manufacturer	Dobon Dinemores, France	Rotam Agrochemical, Hong Kong
Agricultural use	Systemic fungicide used to control many plant diseases including early and late blight of different vegetables such as potatoes and tomatoes as well as the powdery mildews of vitis.	Agricultural systemic fungicide used to control several plant diseases caused by pathogenic fungi on several types of fruits and vegetables including apple scab and powdery mildews of vitis.

mixtures were incubated for 30 min at 28°C. Similar reaction mixtures using distilled water with reagents were used as a blank. 3 ml of Nelson's solution were added and the reaction mixtures were shaken and placed in a boiling water bath for 15 min. After cooling, 3 ml of the arsenomolybdate solution was added, mixed thoroughly and then diluted to l0 ml with distilled water. The whole mixture was centrifuged to remove any turbidity. The amount of reducing sugars produced was estimated by determining the optical density (absorption spectrum) at 700 nm wave length with a spectrophotometer (Spectronic ® GeneSys ™ 2 PC). A standard curve was plotted using aqueous solutions of D-glucose with concentrations from 10 to 100 µg/ml.

Effect of fungicides on pectinase enzyme

Alternaria citri and *A. raphani* which were the most active pectinase producing isolates were cultured on liquid medium containing pectin as substrate. 50 ml of the liquid medium were dispensed into each 250 ml Erlenmeyer conical flasks which were autoclaved for 15 min at 1.5 atm. Different doses of fungicide (50, 100, 200, 300 and 400 ppm active ingradient) were individually added to the sterilized liquid medium under aseptic conditions. Media without fungicide served as control. Each flask was inoculated with two agar mycelial discs (10 mm diameter) of the tested fungal isolates obtained from seven days old cultures. Inoculated flasks were incubated for 6 days at 30°C. Cultures were filtered and the filtrates were centrifuged at 4°C for 15 min at 15000 rpm. The clear supernatants were assayed for pectinase activity. Mycelial dry weights of the tested fungi were also determined.

Assay for pectinase activity

Pectinase activity was assayed spectrophotometrically at 235 nm wavelength according to the method described by Sherwood (1966). The reaction mixture contained 3 ml of 0.1% pectin in 0.05 M Tris-HCl buffer (pH 8) and 0.5 ml culture filtrate. The mixture was incubated at 30°C for 3 h, and then 3 ml of 0.01 N HCl and 1 ml of the reaction mixture were mixed properly. The optical density was measured at 235 nm. One unit of pectinase activity was defined as the amount of enzyme causing an increase in the absorbance of 0.01 in 30 min.

Statistical analysis

Statistical analysis of the data was carried out by one way analysis of variance and the means were separated by Turkey's honest significant difference test using Biostat 2008 statistical analysis program (Copyright © 2001–2009 Analystsoft).

RESULTS

Effect of fungicides on cellulase enzymes

Five doses (50, 100, 200, 300 and 400 ppm, active ingredient) from each of the two fungicides (equation pro and Kema zed) were used to study the effect of fungicides on cellulase production by *A. citri* and *C. spicifer* for exo-β-1,4-glucanase (C_1) and *A. alternata* and *A. citri* for endo-β-1,4-glucanase (C_x) enzyme. Exo-β-1,4-glucanase produced by *A. citri* and *C. spicifer* were significantly inhibited by all doses of the two fungicides used; except in 50 ppm of Kema zed and 50 and 100 ppm of *C. spicifer*; the enzyme production was slightly increased compared to the control. Also, equation pro had more inhibitory effect than Kema zed fungicide on both fungi (Table 2, Figure 1). Mycelial growth of *A. citri* and *C. spicifer* were significantly inhibited by the all doses of the two fungicides and inhibition effect increased with increasing fungicides concentration, except in few cases where the mycelial growth of fungi were promoted by 50 or 100 ppm of Kema zed (Table 2 and Figure 1). Generally, the inhibitive effect of fungicides depends on the type and the dose of fungicides. The inhibitive effect was increased with increasing fungicide concentration (Table 2 and Figure 2). Endo-β-1,4-glucanase produced by *A. alternata* and *A. citri* was significantly inhibited by all doses of the two fungicides, except in 50 ppm dose of kema zed the enzyme production was slightly increased compared to the control. The inhibitive effect of the fungicides was increased by the increasing dose (Table 3 and Figure 3). Mycelial growth of *A. alternata* and *A. citri* were inhibited by all doses of fungicides, except in some cases at 50 ppm dose where the fungal growth were stimulated compared to the control. Generally, the inhibitive effect of the fungicides was increased by increasing the fungicide concentration. The inhibitive

Table 2. Effect of fungicides on growth and exo-β-1,4-glucanase production by *Alternaria citri* and *Cochliobolus spicifer* after 6 days of incubation at 30°C.

Fungicide	Equation Pro				Kema Zed			
	A. citri		*C. spicifer*		*A. citri*		*C. spicifer*	
Dose (ppm)	**E.P**	**D. wt**	**E.P**	**D. wt**	**E.P**	**D. wt**	**E.P**	**D. wt**
0 (control)	5.3	246	5.4	113	5.3	246	5.4	113
50	4.0*	120*	4.9	92*	5.5	284*	6.1*	125*
100	3.1*	109*	3.4*	49*	5.2	253	5.6	109
200	2.6*	85*	2.5*	35*	4.3*	217*	4.5*	93*
300	2.4*	65*	2.2*	26*	3.7*	177*	3.6*	63*
400	1.7*	60*	1.6*	24*	2.9*	137*	2.6*	52*
Inhibition percentage	24.5-67.9	51.2-75.6	9.2-70.4	18.6-78.8	1.9-45.3	11.8-44.3	16.7-51.9	3.5-54.0

E.P = Enzyme production (µg/ml) D. wt = Dry weight (mg/50 ml). Asterisked values mean significant difference from the control.

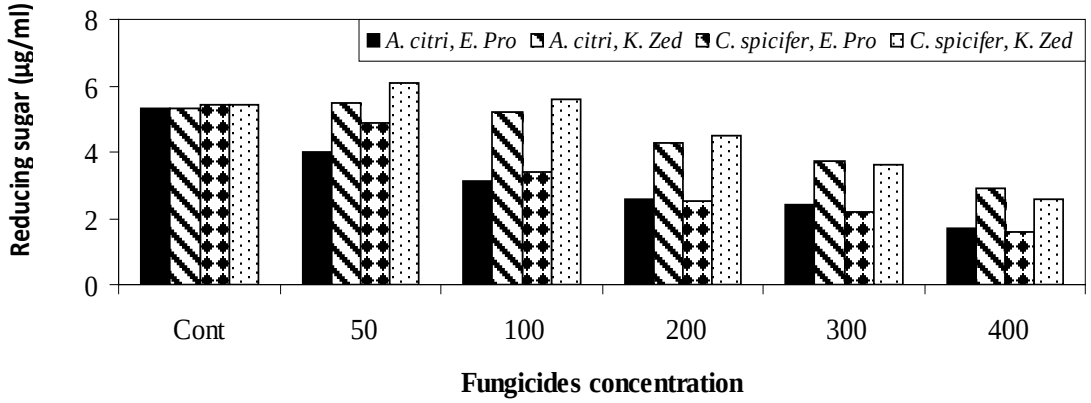

Figure 1. Effect of fungicides on exo-β-1,4-glucanase production by *Alternaria citri* and *Cochliobolus spicifer*.

effect of equation pro on endo- and exo-β-1,4-glucanase production ranged between 5.9 to 70.4% by *A. alternata*, *A. citri* and *C. spicifer*. However, kema zed exhibited an inhibitive effect on cellulases which ranged between 1.9 to 51.9% (Tables 2, 3 and Figures 1 to 4).

Effect of fungicides on pectinase enzyme

Five doses (50, 100, 200, 300 and 400 ppm, active ingredient) from two fungicides (equation pro and Kema zed) were used to study the effect of various concentrations of fungicides, in culture medium, on mycelial growth and pectinase production by *A. citri* and *A. raphani*.

Pectinase produced by *A. citri* and *A. raphani* was significantly inhibited by all doses of the fungicides. Generally, the inhibition effect of these fungicides was increased with the increase of fungicides concentration (Table 4 and Figure 5). Mycelial growth of *A. citri* and *A. raphani* were significantly inhibited by all doses of the two fungicides. Generally, the inhibitive effect of these fungicides on mycelial growth of the tested fungi depends on the type of fungicide and doses used. The inhibitory effect of these fungicides was increased with the increase of fungicide concentration. The inhibitive effect of Equation pro on pectinase enzyme ranged between 20.1 to 75.6% by A. citri and A. raphani while kema zed exhibited an inhibitive effect ranged between 13.5 to 62.8%. The inhibitive effect of these fungicides on the mycelial growth of tested fungi was nearly similar to those for the enzyme production (Table 4 and Figures 5 and 6).

DISCUSSION

In most cases, the equation pro and Kema zed fungicides showed an inhibitive effect on the mycelial growth, cellulase and pectinase enzumes, but with variable degrees which depend on the dose and the type of fungicide. Abdel-Kader et al. (1989) studied the effect of Euparen fungicide on mycelial growth and endo-1, 4 ß-D-

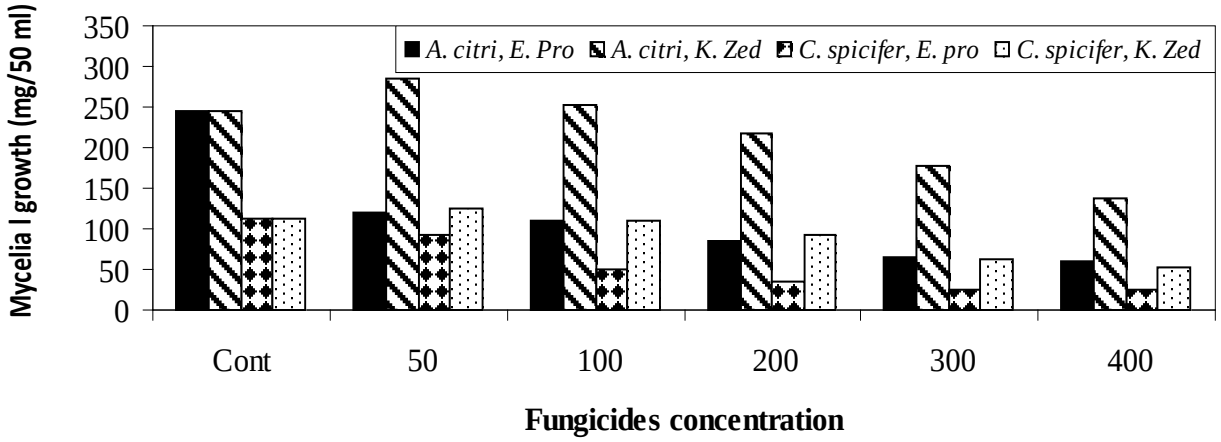

Figure 2. Effect of fungicides on mycelial growth of *Alternaria citri* and *Cochliobolus spicifer*.

Table 3. Effect of fungicides on growth and endo-β-1,4-glucanase production by *Alternaria alternata* and *A. citri* after 6 days of incubation at 30°C.

Fungicide	Equation Pro				Kema Zed			
	A. alternata		*A. citri*		*A. alternata*		*A. citri*	
Dose (ppm)	E.P	D. wt	E.P	D. wt	E.P	D. wt	E.P	D. wt
0 (control)	5.1	165	5.4	139	5.1	165	5.4	139
50	4.8	179*	4.6*	85*	5.0	185*	5.8	141
100	3.7*	145*	4.1*	75*	4.6	151*	4.9*	124
200	3.0*	112*	3.5*	63*	3.8*	132*	4.0*	99*
300	2.5*	70*	2.9*	45*	3.3*	126*	3.6*	94*
400	2.2*	58*	2.6*	41*	3*	119*	3.3*	82*
Inhibition percentage	5.9-56.9	12.1-64.8	14.8-51.8	38.8-70.5	2.0-41.2	8.5-27.9	9.2-38.9	10.8-41.0

E.P = Enzyme production (µg/ml) D. wt = Dry weight (mg/50ml). Asterisked values mean significant difference from the control.

glucanase productions by 11 cellulose-decomposing fungi. The mycelial growth of *Trichoderma viride* was significantly increased by the medium dose and not affected by the other two doses. On the other hand, the mycelial growth and endo-1,4 ß-D-glucanase production of *A. alternata, Fusarium moniliforme*, *Myrothecium verrucaria* and *Thermoascus aurantiacus* were nil at the three doses (4.8, 23.8, 47.5 ppm) of Euparen while *Aspergillus niger* and *Penicillium chrysogenum* were not significantly affected by any dose. The other fungi includes, *A. flavus*, *Chaetomium globosum*, *Drechslera halodes* and *F. solani* which were significantly inhibited or completely eliminated at least by the higher doses. Omar and Abd-Alla (2000) investigated the effect of two fungicides (Afugan and Tilt) at 50 ppm on the activity of various enzymes including cellulase and protease by four nodule colonizing fungi namely *A. awanori*, *A. flavus*, *P. chrysogenum* and *T. koningii*. They found, that, the enzyme activity exhibited different responses to pesticide application. The effect of the two fungicides was essentially stimulatory. Moharram et al. (2004) reported that, the different doses of Kocide caused a regular significant decrease in mycelial growth and cellulase (C_1) production by *A. flavus*, *Cunninghamella echinulata*, *Emercilla* var. *lata*, *F. oxysporum* and *P. aurantiogriseum*. Lower doses of Ridomil plus (50 and 100 ppm) stimulated mycelial growth and cellulase (C_1) production in *A. flavus* and *F. oxysporum.* High doses of Ridomil reduced both fungal growth and enzyme production. Recently, Saleem et al. (2012) studied the effect of Amistar and Moncut fungicides on fungal growth and cellulase production by *A. flavus* var. *columnaris*, *A. fumigatus*, *A. ochraceous*, *Mucor hiemalis* and *T. harzianum*. The incorporation of fungicides in the culture medium for cellulase production exhibited an inhibitive effect on mycelial growth and cellulase production of all fungi by all doses used (100 to 800 ppm).

Mehta (1984) demonstrated that, the maximum reduc-

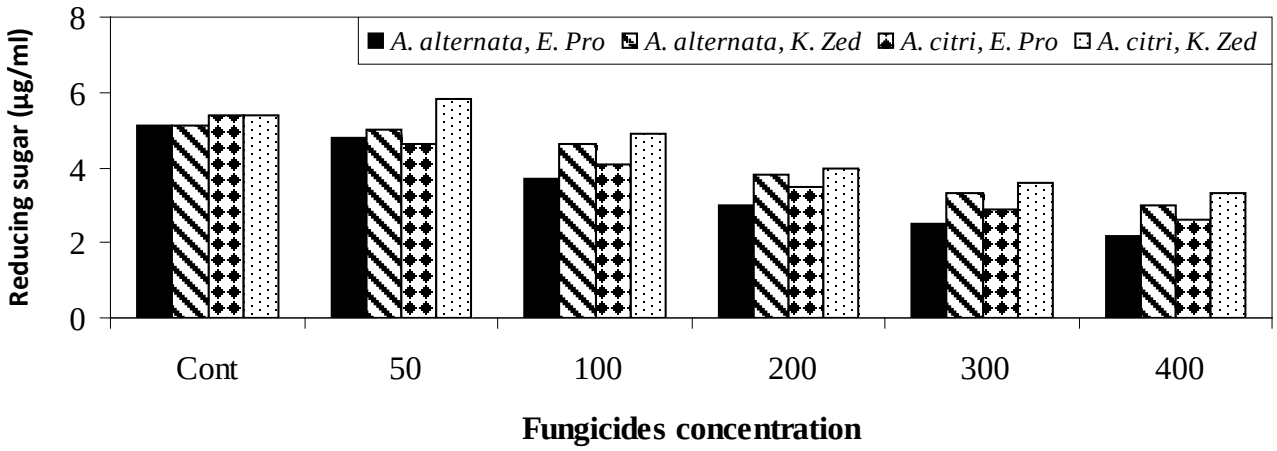

Figure 3. Effect of fungicides on endo-β-1,4-glucanase production by *Alternaria alternata* and *A. citri*.

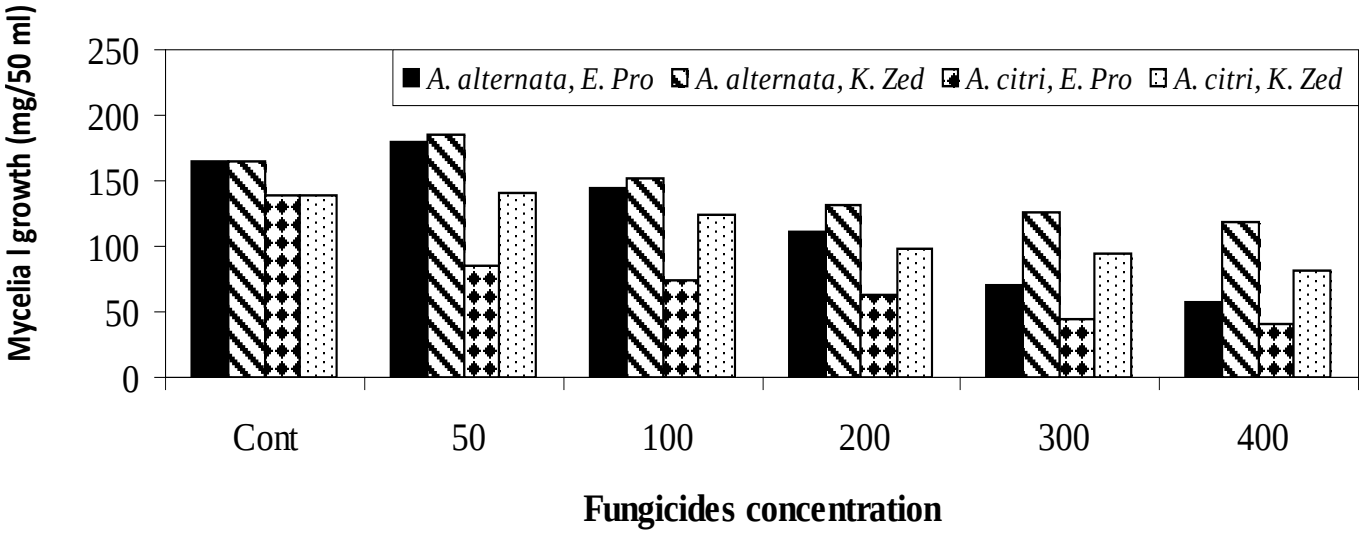

Figure 4. Effect of fungicides on mycelial growth of *Alternaria alternata* and *A. citri*.

Table 4. Effect of fungicides on growth and pectinase production by *Alternaria citri* and *A. raphani* after 6 days of incubation at 30°C.

Fungicide	Equation Pro				Kema Zed			
	A. citri		*A. raphani*		*A. citri*		*A. raphani*	
Dose (ppm)	**E.P**	**D. wt**	**E.P**	**D. wt**	**E.P**	**D. wt**	**E.P**	**D. wt**
0 (control)	2.58	114	2.36	118	2.58	114	2.36	118
50	2.06*	102*	1.79*	85*	2.14*	104*	2.04*	114
100	1.66*	78*	1.59*	58*	1.78*	81*	1.78*	94*
200	1.33*	62*	1.17*	48*	1.52*	65*	1.56*	65*
300	1.02*	51*	0.96*	41*	1.21*	56*	1.14*	63*
400	0.63*	40*	0.65*	34*	0.96*	50*	0.97*	57*
Inhibition percentage	20.1-75.6	10.5-64.9	24.1-72.4	28.0-71.2	17.1-62.8	8.8-56.1	13.5-58.9	3.4-51.7

E.P = Enzyme production (U/ml) D. wt = Dry weight (mg/50ml). Asterisked values mean significant difference from the control.

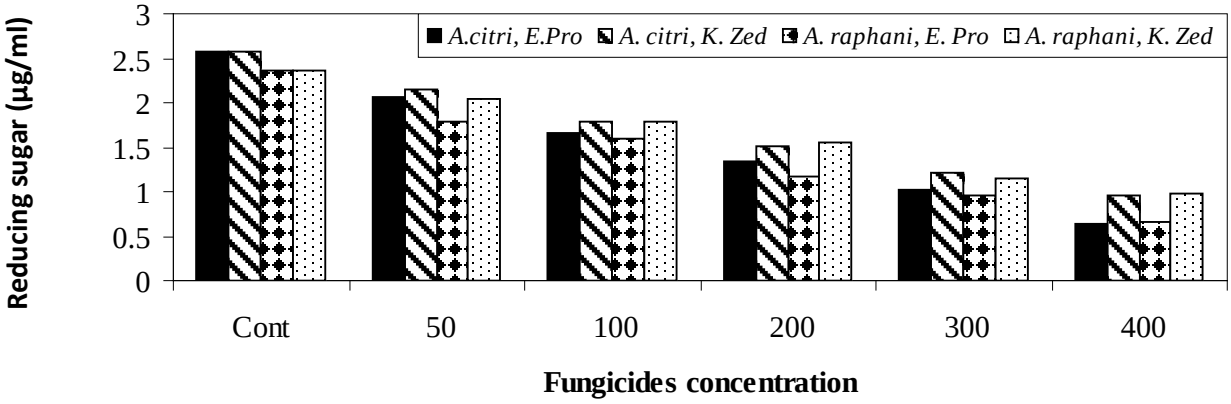

Figure 5. Effect of fungicides on pectinase production by *Alternaria citri* and *A. raphani*.

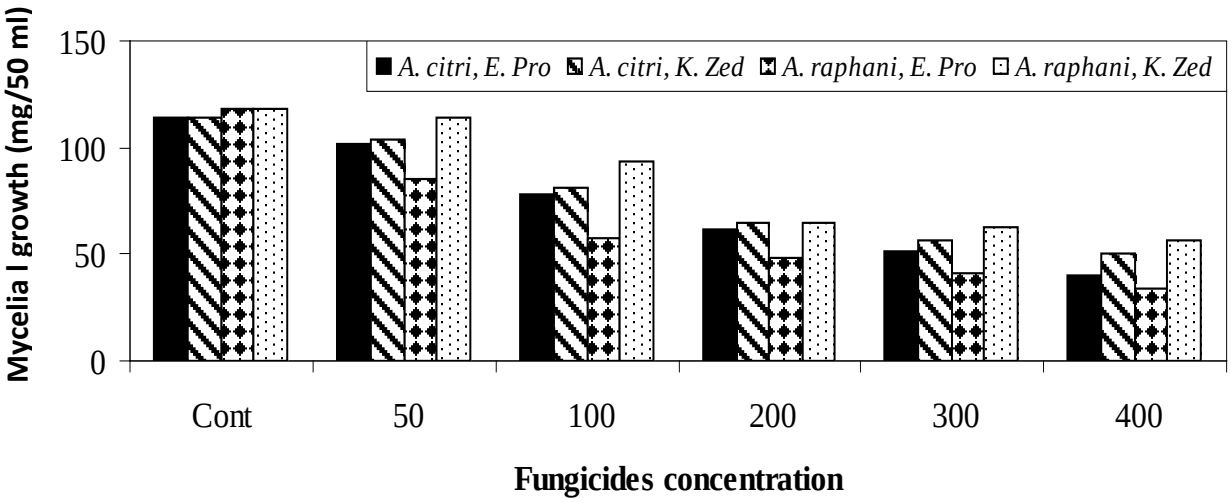

Figure 6. Effect of fungicides on mycelial growth of *Alternaria citri* and *A. raphani*.

tion of pectin methylgalacturonase enzyme synthesis by *A. tenus* was achieved by Brassicol. A high enzyme inhibition was exhibited by Thiram, Cuman, Sultaf and Blimix, respectively. Pectin methylgalacturonase production by *A. tenuis* was also greatly affected by Blitox, Sultaf, Thiram and Brassicol, respectively. Concerning mycelial growth, inhibition of growth was quite variable with different fungicides. Cuman, Blimik and Sultaf showed a stronger inhibition of mycelial growth than Brassicol and Cosan. Blimix was the most effective fungitoxic in nature, other fungicides showed a moderate toxic effect on *A. tenuis*. Gopinath et al. (2006) reported that Propiconazole, Difenoconazole and Carbendazim fungicides inhibited mycelial growth (radial growth and mycelial biomass) of *C. capsici* when compared to controls. Among the fungicides, Propiconazole exhibited the highest level of inhibition, even at lower concentrations. It was followed by Difenoconazole and Carbendazim in descending order. Incorporation of fungicides into the growth medium significantly reduced production of polygalacturonase, polygalacturonase trans-eliminase, pectin trans eliminase and cellulases by *C. capsici*. The highest degree of inhibition was observed in Propiconazole, followed by Difenoconazole and Carbendazim. Recently, Özer et al. (2010) reported that all studied nine fungicides except Triadimenol and Tebuconazole inhibited mycelial dry weight of *Botrytis cinerea* isolates <50%. Cyprodinil + Fludioxonil, Myclobutanil and Imazalil inhibited pectinase activity more than 50%. Fenhexamid had a lower inhibitory effect (<50%) on pectinase activity. Procymidone and Pyrimethanil induced both pectinase activity and isoenzyme banding profile of isolates sensitive to these fungicides.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Vol. 13(46), pp. 4338-4346, 12 November, 2014
DOI: 10.5897/AJB2013. 13424
Article Number: E08AE0648573
ISSN 1684-5315

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African Journal of Biotechnology

Full Length Research Paper

Production of amylase enzyme from mangrove fungal isolates

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Received 27 October, 2013; Accepted 21 October, 2014

The mangrove ecosystem serves as a bioresource for various industrially important microorganisms. The use of fungi as a source of industrially relevant enzymes led to an increased interest in the application of microbial enzymes in various industrial processes. Fungal colonies were isolated from sediments of five different mangrove ecosystems of Odisha such as Bhitarakanika, Dhamra, Mahanadi, Devi and Budhabalanga areas. Forty (40) fungal colonies were isolated and screened for amylase activity out of which five strains were found to be more active. Among the isolates I_{Famy} value was maximum; that is, 5.2 for MSF-9. The amylase enzyme activity of MSF-9 was maximum at pH-5.0, 1% NaCl, 1% substrate and Inositol as carbon source. The most potent fungi was identified through morphological, microscopical and 18S rDNA sequence methods and identified as *Penicillium citrinum*-JQ249898. This strain can be better utilized in large scale industries for enzyme production. Hence further study is suggested on enzyme purification for various value-added product formation.

Key words: Mangrove, fungi, I_{Famy}, *Penicillium citrinum*, microscopical study.

INTRODUCTION

Marine fungi is an important target group for various useful products of industrial importance such as enzymes, sugars, antibiotics, alcohols, beverages, food products etc (Gupta et al., 2007). Study based on screening of fungal resources of mangroves for enzyme and their application has been done to accomplish environment friendly technological development (Maria et al., 2005). Microbial enzymes have completely replaced the chemical hydrolysis of starch in starch process industry (Pandey et al., 2000). Amylases are among the most important enzymes in present-day biotechnology. The enzyme has found numerous applications in commercial processes, including thinning and liquefaction of starch in alcohol, brewing and sugar industries. α-Amylases are hydrolytic enzymes that are widespread in nature, being found in animals, microorganisms and plants (Octávio et al, 2000). In fungi, detailed studies on α-amylase purification have largely been limited to a few species of fungi (AbouZeid, 1997; Khoo et al., 1994). The major advantage of using microorganisms for production of amylases is in economical bulk production capacity and microbes are also easy to manipulate to obtain

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enzymes of desired characteristics (Lonsane and Ramesh, 1990).

In the present study fungal strains were isolated from five different mangrove ecosystems of Odisha, India. The screening of the fungal isolates and enzyme assay of the most active strain will be carried out. Finally the identification of potent fungal strain was done by morphological, microscopical and molecular study.

MATERIALS AND METHODS

Collection of sediment

Sediment samples were collected from five different mangrove ecosystems such as Bhitarakanika, Dhamra, Mahanadi, Devi and Budhabalanga areas of Odisha, India. Sediment samples were collected with an air dried Beckman's grab. The sediment collected were aseptically transferred to labeled polythene bags and kept in an ice-chest box before transferring to the laboratory. The collected sediments were air dried for 7-10 days at room temperature in the laboratory for further study.

Isolation of microorganism

Isolation of fungi has been was done through serial dilution technique by taking one gram of air-dried sediment samples. The air-dried sediment sample was serially diluted with 50% sea water and plated on the Potato Dextrose Agar (PDA) media (Himedia) by spread plate method with the addition of 100 mg/L of ampicillin to avoid unwanted growth of bacteria and the culture plates were incubated at 28°C for 36-48 h. The total viable count (TVC) was estimated from the mixed culture plate. The pure culture was obtained by repeated sub-culturing of the fungal isolates from the mix culture plates. Then these pure cultures were maintained in fresh PDA slants and preserved at 4°C for further study.

Amylase enzyme assay

Screening of isolates

Primary screening was done by using 1% of starch soluble in the media plate. The results were recorded by using Gram's iodide in the agar plate and the strains showing hydrolysis were selected and index value (I_{Famy}) has been determines for the isolates.

Production medium

The production medium used for growth of the fungal isolate was soluble starch 50 g, yeast extract 0.5 g, KH_2PO_4 10 g, $(NH_4)_2SO_4$ 10.5 g, $MgSO_4$ 0.3 g, $CaCl_2$ 0.5 g, $FeSO_4$ 0.013 g, $MnSO_4$ 0.004 g, $ZnSO_4$ 0.004 g, CoCl 0.0067 g, 50% seawater. The pH was adjusted to 6.5 and the media were sterilized in an autoclave for 15 min at 121°C. The media were inoculated with a loop-full of fungal spore suspension and incubated at 30°C in an orbital shaker set at 100 rpm for 72 hrs. The media were centrifuged at 5,000 g for 15 min to obtain crude enzyme solution.

Enzyme assay

Amylase assay has been was carried out by taking the reaction mixture (4 mL) which consisted of 1 mL of centrifuged enzyme solution and 2 mL of soluble starch in phosphate buffer, pH 6.5 (Wood and Bhat, 1988). The mixture was incubated for 10 min at 30°C. The amount of reducing sugars released was determined by dinitrosalicylate method (Miller, 1959) at 540 nm and is expressed in units (one unit is the amount of enzyme releasing 1 mg of glucose per mL per minute).

Optimization of culture conditions

The factors such as pH, salinity, carbon sources and different substrate conc. concentrations and affecting production of amylase were optimized by varying the parameters one at a time. The experiments were conducted in 250 mL Erlenmeyer flask containing 150 mL of production medium for each parameter. After sterilization by autoclaving, the flasks were cooled and inoculated with culture and maintained under various culture conditions such as pH (3.0, 5.0, 7.0, 9.0 and 11.0), carbon source (D-glucose, lactose, dextrose, sucrose and inositol at 1%), salinity (0, 0.5, 1, 1.5, 2.0 and 2.5% of NaCl) and substrate conc. (0, 0.5, 1, 1.5, 2.0 and 2.5% of starch). After 72 hr (expect for incubation period effect), 4 mL of the culture filtrate was assayed in triplicate to study the enzyme activity.

Statistical analysis

All experiments were carried out in triplicates, and repeated three times. The samples collected from each replicate were tested for amylase production and activity. Means and standard errors of amylase activity and production were calculated, respectively. and significant differences were calculated by determining standard error. The significance level of the individual parameters was done through The data were statistically analyzed using one way analysis of variance (ANOVA) and Tukey's multiple comparision test with the aid of the software, Graph pad 5.0; data with p-values lesser than 0.05 were considered significant.

Identification of potent strains

Morphological analysis

The fungal isolates were identified by studying the morphological and microscopical study. Morphological characterization was carried done by studying the upper and lower surface of the culture plate.

Microscopical analysis

The microscopical analysis has been done by using Lactophenol cotton blue (LPCB) staining method and were observed under compound microscope. The fungal spores were also observed under the SEM to observe the spore surface and structure.

Molecular taxonomy of the potent isolates

Phylogenetic (18S rDNA) analysis of the strain the potent the most amylase-producing strain possessing maximum I_{FAMY} value was carried out using following procedures

Genomic DNA isolation

Genomic DNA was isolated by using approximately 50mg of mycelium (wet weight) from a fresh culture plate as described by Hapwood et al. (1985).

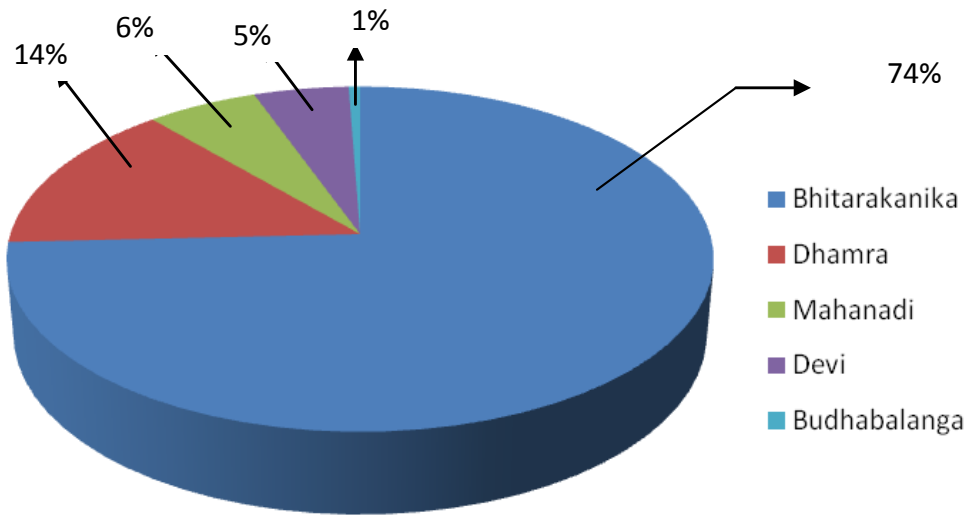


Figure 1. Comparative study of fungal diversity of mangrove ecosystems of Orissa.

PCR amplification of the 18S rDNA

Genomic DNA from the fungal cultures was used to perform PCR reaction. The 500bp of rDNA fragment was amplified using high fidelity PCR polymerase. 10 µL of the amplifications were analyzed on 1% agarose gel.

18S rDNA sequencing

The purified fragments were directly sequenced bidirectionally. The data was analyzed using applied biosystem DNA editing and assembly software and sequence comparisons were obtained using the micro Seq Software.

Sample identification

A distance matrix was generated using the Jukes-Cantor corrected distance model. Identification was done on the pair wise alignment algorithms and phylogenetic tree.

Phylogenetic analysis

Sequence similarity search was made for the 18S rDNA sequence of the fungal strain MSF-9 by applying its sequence to BLAST search for in NCBI. The Evolutionary tree was inferred by using neighbor-joining method (Thompson et al., 1997). The CLUSTAL X package was used for multiple alignment and identification of the strains.

RESULTS AND DISCUSSION

Diversity analysis

The total fungal diversity was found to be maximum that is, 74% at Bhitarakanika, 14% at Dhamra, 6% at Mahanadi, 5% at Devi and least 1% at Budhabalanga mangrove sediments of Orissa (Figure 1).

Amylase screening

Maximum index value I_{Famy} was observed in case of MSF-9 that is, 5.2 whereas in MSF-3, MSF-7, MSF-13 and MSF-28 it was 3.09, 2.68, 3.63 and 4.75, respectively (Figure 2).

Amylase assay

Effect of pH

The most potent amylase-producing fungal culture (MSF-9) was inoculated into the flask containing amylase assay media at different pH such as 3.0, 5.0, 7.0, 9.0 and 11.0. The enzyme activity was maximum at pH-5.0, that is, 80.68±0.09U mL^{-1} whereas minimum activity; that is, 22.67±0.33 U mL^{-1} was observed at pH-3.0. At pH-7.0 and 9.0 the enzyme activity was 67.44±0.23 U mL^{-1} and 58.58±0.41 U mL^{-1} respectively. The enzyme activity was first increased up to pH 5.0 and then gradually decreased with the increase of pH (Figure 3).

Effect of NaCl

The isolate MSF-9 was inoculated into the flask containing amylase assay broth at different NaCl concentrations such as 0, 0.5, 1, 1.5, 2 and 2.5%. The enzyme activity was maximum at 1% NaCl; that is, 36.89±0.26 U mL^{-1}, whereas minimum activity (6.76±0.063 U mL^{-1}) at 0% NaCl. At various concen-

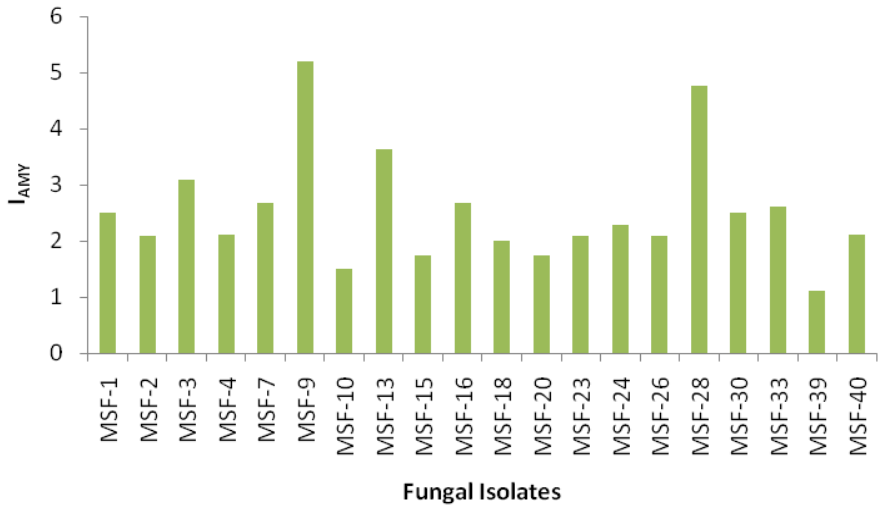


Figure 2. Screening for amylase enzyme activity of mangrove fungal isolates.

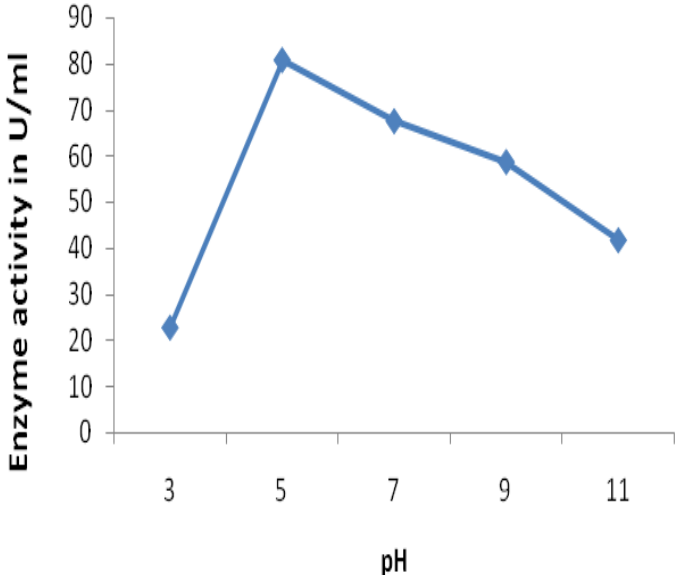


Figure 3. Effect of pH on amylse enzyme activity of MSF-9 strain.

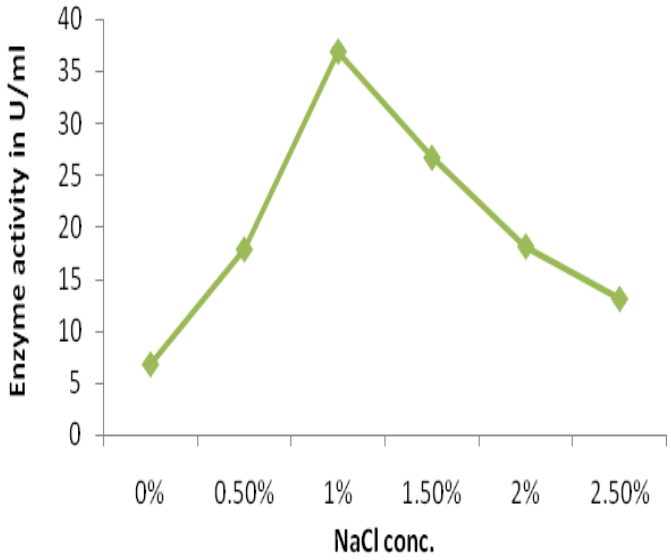


Figure 4. Effect of NaCl conc. (in %) on amylse enzyme activity of MSF-9 strain.

trations of NaCl such as 0.50, 1.50, 2 and 2.5%, the enzyme activity was 17.84±0.14 U mL^{-1}, 26.66±0.18 U mL^{-1}, 18.13±0.38 U mL^{-1} and 13.06±0.30 U mL^{-1} respectively. The enzyme activity first increased up to 1% NaCl and then gradually decreased (Figure 4).

Effect of substrate concentration

The selected strain MSF-9 was inoculated into the flask containing amylase assay broth at different substrate levels such as 0, 0.5, 1, 1.5, 2 and 2.5%. The enzyme activity was maximum at 1% starch soluble; that is, 98.25±0.23 U mL^{-1}, whereas minimum activity (12.64±0.711 U mL^{-1}) at 0% substrate. At various concentrations of 0.50, 1.50, 2 and 2.5%, the enzyme activity was 49.02±0.54 U mL^{-1}, 65.42±0.45 U mL^{-1}, 54.86±0.69 U mL^{-1} and 45.51±0.34 U mL^{-1} respectively. The enzyme activity first increased up to 1% starch soluble and then gradually decreased (Figure 5).

Effect of carbon sources

The amylase enzyme activity of MSF-9 was recorded at

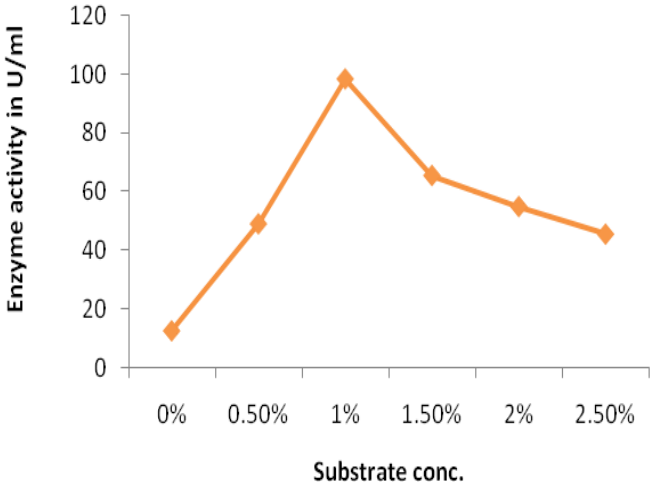


Figure 5. Effect of substrate conc. (starch) on amylse enzyme activity of MSF-9 strain.

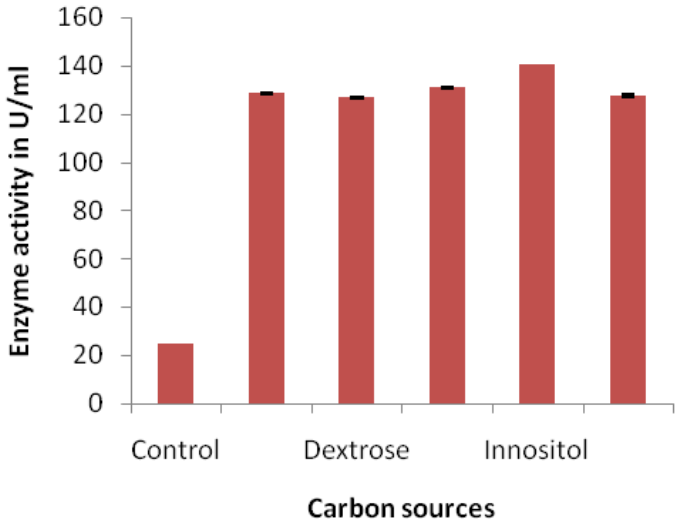


Figure 6. Effect of carbon sources on enzyme activity.

different carbon sources such as D-glucose, Dextrose, Sucrose, Inositol, and Lactose. The enzyme activity of MSF-9 was found to be minimum in lactose (127.58±0.754 U mL^{-1}) but maximum in inositol (140.58±0.22 U mL^{-1}). In case of control, D-glucose, dextrose and sucrose, the activity was 24.84±0.05 U mL^{-1}, 128.664±0.39 U mL^{-1}, 126.74±0.079 U mL^{-1}, and 131.042±0.079 U mL^{-1} respectively (Figure 6).

Significant relationship between individual of variables such as pH, NaCl conc. concentration, substrate concentration and carbon sources was observed through the one way analysis of variance (ANOVA) and Tukey's multiple comparison tests ($P<0.05$).

Identification

Morphological and microscopic analysis

Morphological study of the fungal isolates was carried out by growing the isolates on PDA growth media. Among the forty different isolates, twenty isolates such as MSF-1 to MSF-20 were found commonly in all the five study areas. The twenty strains were further characterized and screened for the presence of amylase enzyme. Out of the 20 isolates, five strains (MSF-3, MSF-7, MSF-9, MSF-13 and MSF-28) were found to possess more activity after screening and were observed under microscope by using cotton blue stain (Figure 2). The growth patterns of the potent fungal isolate; that is, MSF-9 was carried out in different media such as PDA, CDA (Czapek Dox Agar) and MA (Mycological Agar). It was showing very good growth in CDA medium than other two media (Table 1 and 2). The mycellial structure observed under microscope and identified (Figure 7).

SEM Study

The dried aerial mycelium with spore of the potent fungal strain (MSF-9) was studied by cover slip culture method in the SEM (Zeiss). The spores were attached like woolen balls and the round balls like structures forms a chain which observed at different magnifications from low to high; that is, 4.21K×10, 000, 8.78 K×10,000, 23.98 K×10,000 and 29.32 K×10,000 at 2.00 KV. From the spore structures it was assumed to be the member of the genus *Penicillium* (Figure 8).

Molecular identification of the potent most productive isolate (MSF-9)

The potent most productive fungal isolate, MSF-9, was identified by molecular methodology. Partial 18S rDNA sequence having up to 555 bp was amplified by PCR. The similarity pattern of the target sequence was compared with other sequences in NCBI database. The similarity pattern of the target isolate was compared with other strains and it was showing showed 97% significant homology with *Penicillium citrinum* GZU-BCECYN60-2 by using the universal marker; that is, F-27 and R-1492. The band pattern of the PCR product of MSF-9 resembles 97% with of the strain *Penicillium citrinum* (Table 4).

Phylogenetic tree

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). From the tree it can be confirmed that the isolate belongs to *Penicillium citrinum* (Figure 10).

Table 1. Morphological and microscopic characteristics of the fungal isolates.

Culture	Morphological characteristics
MSF-3	Black colony with whitish margin, smooth, reverse light yellow
MSF-7	Colonies growing rapidly up to 9cm in four days, whitish, yellowish green
MSF-9	Pale yellowish brown colony, white periphery, reverse light brownish, medium to small in size
MSF-13	White at first becoming pinkish later on, small to medium colony
MSF-28	Greenish, slow growing, reverse pale yellow in colour

Table 2. Identification of fungal Isolates showing maximum amylase enzyme activity.

Given names	ID No.	Identified strains
MSF-3		*Aspergillus niger*
MSF-7	3724.10	*Trichoderma viride*
MSF-9	3728.10	*Penicillium citrinum*
MSF-13	3731.10	*Paecilomyces variotii*
MSF-28	4108.10	*Eurotium amstelodmi*

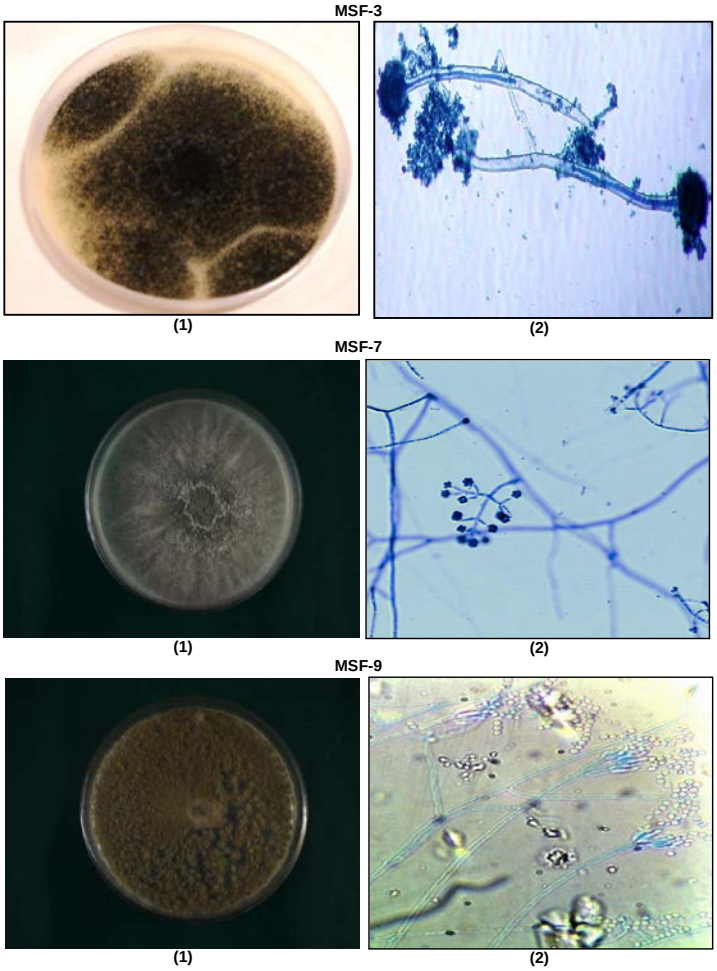
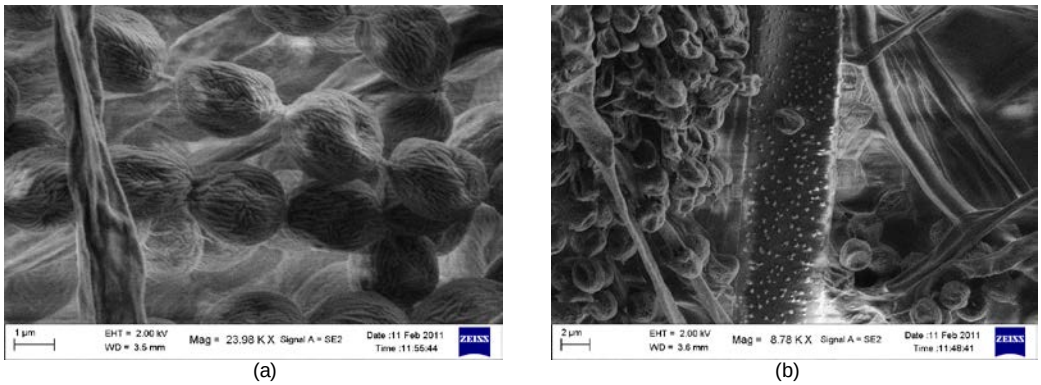


Figure 7. Pure culture ((1)) and respective microscopic photo ((2)) of MSF-3, MSF-7, MSF-9, MSF-13 and MSF-28 fungal isolates



(a) (b)

Figure 8. SEM photos of the fungal isolate (MSF-9) having magnification at 23.98 K×10,000 (a) and 8.78 K × 10, 000 (b).

Table 3. Growth characteristics of MSF-9 in different media.

Media Used	Growth characteristics
PDA	Good growth (Upper part- Light brownish, lower part- off white or light orange, pigment- No)
CDA	Very good (Upper part- Light brownish, lower- yellowish black, Pigment- yellow to black soluble)
MA	Slow growth (Upper part- Light brownish, lower- yellowish black, Pigment- No)

PDA; CDA; MA, Mycological agar

DISCUSSION

Distribution of fungal species within the mangrove habitat may reflect the physical conditions and/or habitat preference such as temp., salinity, humidity, organic contents (Das et al., 2008; Ravikumar et al., 2004). The diversity was maximum at Bhitarakanika that is, 74% and least 1% at Budhabalanga mangrove sediments which may be due to the clayey sediment which indicates the presence of more humus content.

Selections of new microorganisms for enzyme production are increasing all around the world. So the study site is mainly focused on mangrove areas of Odisha, India which are very less explored. The major advantage of using microorganisms for production of amylases is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics (Lonsane and Ramesh, 1990). Starch-degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food fermentation, textile to paper industries (Lin et al., 1997; Pandey et al., 2000). The selection of potent strains for enzyme production was done by screening and calculating the index values which indicates the utilization of substrate by the mangrove isolates. During screening of amylase enzyme activity, the fungal isolate *Penicillium citrinum* has maximum I_{Famy} value 5.2 and selected for enzyme assay purpose. The media optimization is an important aspect to be considered in the development of fermentation technology. However, there are only a few reports concerning the optimization of media composition for fungal strains in amylase production (Quang et al., 2000). The potent fungal strain That is, *Penicillium citrinum* from the present study was found to have better activity in all the different optimized culture conditions. The maximum amylase production for the strain was found to have maximum enzyme activity at pH-5.0, 1% NaCl, 1% of substrate (starch) and Inositol as carbon source. Similar finding has been done by Kathiresan and Manivannan (2006) for fungi. Similar type of study was conducted by Gupta et al. (2010) where maximum enzyme production has been observed for the fungal strain *Aspergillus niger.* The pH change observed during the growth of microbes also affects product stability in the medium (Gupta et al., 2003). Most of the earlier studies revealed the optimum pH range between 5.0 and 7.0 for the growth of some fungal strains such as *P. fellutanum,* however, *Aspergillus oryzae* released amylase only in alkaline pH above 7.2 (Yabuki et al., 1977).

Identification of fungi was carried out by the usual methods such as cotton blue staining and conidia or spore structure attachment etc. The two genus such as *Aspergillus* sp., and *Penicillium* sp. were found to be dominant in the present study area, which were expected to furnish optimal conditions for the discovery of new metabolites from mangrove associated fungi. The fungal strains were identified as *A. niger, Trichoderma viride, Penicillium citrinum, Paecilomyces variotii, Eurotium amstelodmi* by microscopic and morphological observation (Table 3 and Figure 7). *Penicillium citrinum*

Table 4. Alignment view and distance matrix of the MSF-9 isolate.

Accession	Description	Max. score	Total score	Query coverage (%)	E value	Max ident (%)
GU565136	*Penicillium citrinum* GZU-BCECYN60-2	795	795	85	0	97
HQ407424	*Eupenicillium brefeldianum* TZ-16	728	728	85	0	94
HM214448	*Penicillium janthinellum* Zh9A	728	728	85	0	94
GU981580	*Eupenicillium brefeldianum* CBS:235.81	728	728	85	0	94
AF033443	*Penicillium fuscum* NRRL 721	728	728	85	0	94
AF033435	*Eupenicillium brefeldianum* NRRL 710	728	728	85	0	94
HM469409	*Penicillium* sp. 6 JJK-2011	725	725	86	0	94
FJ613818	Fungal endophyte sp. ZY-2009	725	725	85	0	94
AM262422	*Eupenicillium* sp. SS-1627	725	725	83	0	94
AF481123	*Penicillium* sp. NRRL 28214	725	725	86	0	94

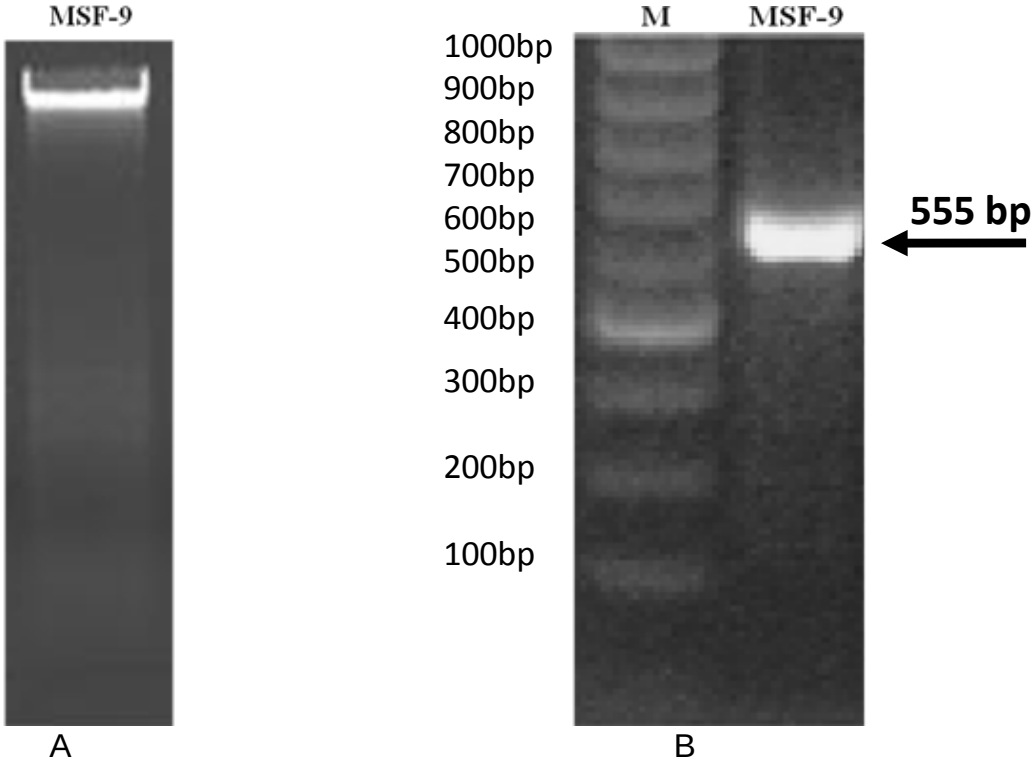


Figure 9. Agarose gel electrophoresis of the genomic DNA (A) and 18S rDNA PCR Product (B) of MSF-9 (M- 100bp Marker).

strain was found to grow luxuriantly in CDA media and the SEM study indicates the general Penicillium. The molecular study that is, 18S rDNA analysis showed that the MSF-9 isolate has 96% similarity with *Penicillium citrinum* GZU-BCECYN60-2 (Table 4, Plate 35). The phylogenetic analysis showed the similarity pattern of *P. citrinum* (MSF-9) with other species (Figures 9 and 10). It can be concluded from the present study that the mangrove isolate *P.citrinum* produced more amylase enzyme. Hence this potent fungal strain can also be used in large scale industry for various useful product formation.

Conflict of Interests

The author(s) have not declared any conflict of interests.

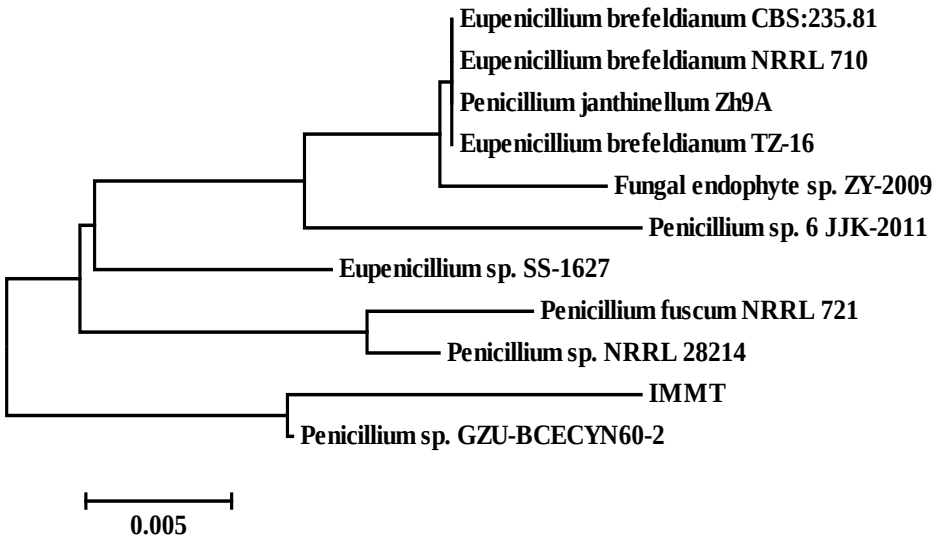


Figure 10. Evolutionary relationships of 11 taxa of MSF-9.

ACKNOWLEDGEMENTS

The authors are thankful to the Director, CSIR- IMMT, Bhubanes war for providing necessary facilities to carry out the work. The corresponding author is very much thankful to Dr. P. N. Chowdhry, NCFT, New Delhi for identification of fungi.

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Vol. 13(46), pp. 4347-4354, 12 November, 2014
DOI: 10.5897/AJB2014.13683
Article Number: C49FDC548575
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Antimicrobial activity and chemical analysis of some edible oils (Clove, Kalonji and Taramira)

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Received 31 January, 2014; Accepted 21 October, 2014

Antimicrobial activity of oils of *Syzigium aromaticum* (Clove), *Nigella sativa* (Kalonji) and *Eruca sativa* Miller (Taramira) was checked against bacteria and fungi by agar well diffusion assay. It was found that Gram negative bacteria were more sensitive to these oils as compared to Gram positive bacteria. *Klebsiella pneumonia, Aspergillus flavus* and *Cunninghamella* were found to be more sensitive organisms showing large zones of inhibition. Taramira showed highest antifungal potential as compared to other tested oils. Further, oils were also checked in combination with each other and with antibiotics but no significant results were obtained. Activity units of Kalonji, Taramira and Clove oils were found to be 160, 20 and 160 AU/ml, respectively. Clove and Kalonji oils have higher MICs (that is, 1:4) as compared to Taramira oil (that is, 1:2). Effects of *S. aromaticum* and N. sativa oils on growing cells of *K. pneumonia* were found to be bactericidal. Moreover oils were chemically analyzed to determine their composition where moisture content was found to be 9.22% for Clove, 7.79% for Kalonji and 12.25% for Taramira. Acid values of Clove, Kalonji and Taramira oils were found to be 0.418, 1.673 and 0.334%, respectively whereas iodine values of Kalonji and Taramira oils were found to be 23.173 and 41.950 ml, respectively. Current study was focused to investigate the antibacterial and antifungal potential of natural edibles that is, Clove, Kalonji and Taramira oils in order to treat infectious diseases caused by bacteria and fungi. Taramira can be used to treat fungal infections as it exhibits maximum activity against tested fungi. Thus we can conclude that these oils can be used as bio control agents to treat bacterial and fungal infections.

Key words: Antimicrobial activity, Clove, Kalonji, Taramira, chemical analysis.

INTRODUCTION

Many developed as well as developing countries are facing problems related to infectious diseases that cause mortality and morbidity (Lewis et al., 2006). Antibiotics can cause alterations in intestinal microecology leading to pathogen colonization and over growth (Sullivan et al., 2001; Hooker et al., 1988). Overuse of antibiotics,

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incomplete course of treatment and horizontal transfer of resistant genes among bacterial species reduce the effectiveness of antibiotics (Amit et al., 2006; Aibinu, 2007). Earlier study described that the administration of antibiotics may cause disturbance of gut microbiota (Clayton et al., 2006).

All these factors forced the researchers to search for alternative antimicrobial substances from different sources including plants and plant based products (Mandal et al., 2010).

Natural products have been an integral part of ancient traditional medicine systems for example, Chinese and Egyptian (Sarker and Nahar, 2007). Nigella sativa is a small herbaceous plant, one of the Ranunculaceae, contains 40% saponin (melanthin) and up to 1.4% volatile oil (Chevallier, 1996).

Traditional medicine uses N. sativa seed and its oil for the treatment of different illnesses including bronchial asthma in adults. These seeds have been used traditionally for long time in the Middle East, Northern Africa and South Asia for the treatment of various diseases (Brutis et al., 2000). It is used as laxative, carminative and intestinal antiprotozoal drug (Amin, 1990). Their crude extracts (Ali et al., 2001; Mouhajir et al., 1999) and essential oil (Halwani et al., 1999) possess antibacterial.

Clove oil can be obtained from the flower buds of Syzigium aromaticum, family Myrtaceae. Its active ingredient 'eugenol' bears antimicrobial properties against many bacteria like *Campylobacter jejuni, Salmonella enteritidis*, L. *monocytogenes, Escherichia coli* and *Staphylococcus aureus* (Beuchat, 2000; Cressy et al., 2003) and inhibits the growth of molds and yeasts (Matan et al., 2006). Clove oil is used as a food flavoring agent (Zheng et al., 1992), as a medicine for the treatment of asthma and different allergies (Kim et al., 1998), and as an antiseptic in medical dental practices (Cai and Wu, 1996). Lee and Shibamoto (2001) reported that Clove oil has antioxidant activity and can also be used as an anti-carcinogenic agent. Eruca sativa, also known as rocket or arugula, is an edible plant. It is an annual plant growing to 20-100 cm tall and their flowers are 2-4 cm in diameter.

It is rich in vitamin C and potassium. E. sativa Miller grows in Middle East, India and Pakistan as a minor oil crop and it is used for the preparation of traditional medicines (Flanders and Abdul Karim, 1985). Their seeds can yield oil up to 35% (Yadava et al., 1998) and 27% of protein (Flanders and Abdul Karim, 1985). It resists drought conditions, possesses the ability of salt tolerance (Shannon and Grieve, 1999) and commonly used as animal feed in Asia (Kim and Ishii, 2006). Current study was focused to investigate the antibacterial and antifungal potential of natural edible oils that is, Clove, Kalonji and Taramira oils in order to treat infectious diseases caused by bacteria and fungi.

MATERIALS AND METHODS

Microorganisms and culture conditions

Eighteen (18) bacterial and eight fungal cultures were collected from Reference Culture Collection Laboratory (RCCL) of Department of Microbiology, University of Karachi to check the antimicrobial activity. Their purity was checked on the basis of standard microbiological characteristics as per Bergey's Manual of Determinate Bacteriology 9th edition by Holt (1994). Bacterial cultures were maintained in nutrient agar (NA) and fungal cultures were maintained in Sabraud dextrose agar (SDA) slants at 4°C.

Crude oils and standard antibiotics

S. aromaticum (Clove), *N. sativa* (Kalonji) and *E. sativa* Miller (Taramira) oils were purchased locally from markets in Karachi, Pakistan. Equal volume of absolute methanol was added into oils in order to get better diffusion (Pretorius et al., 2003). Standard antibiotics ampicillin, streptomycin, chloramphenicol and cefamezin were used for antibacterial activity and nystatin was used for antifungal activity.

Standardization of microorganisms

Duly isolated colonies from 18-24 h culture were picked and transferred into tube containing 3 ml phosphate buffer saline pH 7 (Roopashree et al., 2008). Density of the suspension was adjusted to 0.5 McFarlane standards.

Screening for antimicrobial activity

Agar well diffusion method was followed for screening of antibacterial and antifungal activities of oils. Briefly, following standardization and making lawn on Muller Hinton agar (for antibacterial activity) and SDA (for antifungal activity); wells were cut in agar and 30 µl of methanol diluted oils (1:1) were placed. 30 µl of methanol was placed in a well serving as a control. MHA plates were incubated at 37°C for 24 h and SDA plates were incubated at ambient temperature for 48-72 h and observed for the zones of inhibition around each well as described by Adwan et al. (2008).

Determination of combined effect

In order to determine the combined effect of oil with oil, equal volume of two oils were mixed and incubated for 1 h prior to determine antibacterial and antifungal activities as described earlier. Plates were incubated at 37°C for 24 h (for bacteria) and at ambient temperature for 48-72 h (for fungi). Same protocol was followed in order to determine the combined effect of oils with antibiotics.

Determination of activity unit (AU/ml)

Modified method of Mayr-Hartings et al. (1972) was followed to determine the activity units of oils on MHA medium by agar well diffusion method. Oils were serially diluted by using methanol and transferred to wells made on plates seeded with standardized inoculum of *Klebsiella pneumonia*. Activity unit (AU/mL) is defined as the reciprocal of the last serial dilution demonstrating inhibitory

activity after an incubation period.

Determination of minimum inhibitory concentration (MIC)

In order to determine MIC, oils were serially diluted by using methanol and 20 µl of standardized inoculum of *Klebsiella pneumoniae* was transferred to each tube, incubated at 37°C for 24 h to observe turbidity (CLSI, 2011). MIC is defined as the reciprocal of the last serial dilution demonstrating no turbidity after an incubation period.

Mode of action of oils

Modified method of Bhunia et al. (1988) was followed in order to evaluate mode of action of screw pressed Clove and Kalonji oils on the viable count of log phase cells. Briefly, sensitive culture was inoculated into BHI broth and incubated at 37°C under shaking condition for 2 h to get the log phase culture. 100 µl of this log phase culture was then transferred in the tube containing screw pressed oil and incubated at 37°C. After every hour, 500 µl of sample was taken out and optical density at 600 nm was measured. Control was run simultaneously by using BHI broth.

Chemical analysis

Clove, Kalonji and Taramira oils were chemically analyzed simultaneously in three parallel reactions as described by American Oil Chemists Society (AOCS, 1998):

Moisture content

Oil was placed in hot oven at 105°C for 2 h followed by reweighing. Difference in weight indicates moisture content.

Free fatty acids (FFA) and acid value (AV)

Oil samples were filtered through filter paper in order to remove any debris. Equal volume of diethyl ether was mixed with alcohol and neutralized with 0.1 N NaOH in the presence of phenolphthalein. Oil was dissolved in neutral solvent and titrated with aqueous 0.1 N NaOH till the appearance of pink color.

Calculation:

FFA values are usually reported as oleic acid; however, in oils such as coconut and palm kernel, FFA is reported in terms of lauric acid or palmitic acid, respectively.
For oleic acid, FFA was calculated as follows:

$$\text{FFA as \% oleic acid} = \frac{\text{ml NaOH} \times \text{NaOH normality} \times 28.2}{\text{weight of sample (g)}}$$

Acid value was calculated as follows:

FFA × 1.99 for oleic acid
FFA × 2.81 for lauric acid
FFA × 2.19 for palmitic acid

Iodine value (IV)

Oil samples were filtered through filter paper and placed in water bath at 70°C. Cyclo-hexane was added and dissolved into the oil followed by Wijs' solution. Flask was sealed and placed in dark for 1 to 2 h. 10% potassium iodide (KI) solution and distilled water were added. Titration was done with 0.1 N sodium thiosulphate till the disappearance of yellow-brown color.

Soluble starch was added and then titration was continued until the disappearance of blue/brown color. Blank was run with cyclo-hexane simultaneously. Calculation was done by using following formula.

Calculation:

$$\text{IV} = \frac{\left(\text{ml } Na_2S_2O_3 \text{ for blank} - \text{ml } Na_2S_2O_3 \text{ for sample}\right) \times Na_2S_2O_3 \text{ normality} \times 12.69}{\text{sample weight (g)}}$$

RESULTS

Antimicrobial activity

S. epidermidis was the most resistant organism among Gram positive bacteria and showed zone of inhibition only against streptomycin with 27 mm diameter. Klebsiella pneumonia was found to be more resistant organism among gram negative bacteria and showed no zone of inhibition against any of the standard antibiotics (Tables 1, 2, 3 and 4). Moreover, maximum antibacterial activity was achieved by Clove and Taramira oils against E. *coli* 5014 and by Kalonji against *K. pneumoniae*.

Antifungal activity of Clove, Kalonji and Taramira oils by using agar well method is depicted in Table 5. Kalonji oil showed no antifungal activities while Clove showed comparatively low antifungal activities against tested fungi. Taramira exhibited maximum activity against Nigrosporum with 40 mm diameter of zone of inhibition and it was found that this oil possessed anti dermatophytic activity.

Combined effect

In current study, oils were also checked in combination with each other and with antibiotics but no significant results were obtained. Combination of oils with each other and with antibiotics antagonized the antimicrobial potentials of the tested oils (Figure 1).

Activity units and MIC

It was found that Clove and Kalonji oils have higher activity units and MICs as compared to Taramira oil against *Klebsiella pneumoniae* as shown in Figures 2, 3 and Table 6.

Mode of action

Effects of screw pressed *S. aromaticum* (Clove) and N.

Table 1. Antibacterial activity of *Syzigium aromaticum* (Clove), *Nigella sativa* (Kalonji) and *Eruca sativa Miller* (Taramira) oils and rice bran (against gram positive bacteria).

Organism	Zones of inhibition (in mm)			
	T	K	C	M
Staphylococcus aureus	-	15	14	12
Staphylococcus epidermidis	12	16	25	20
Bacillus subtilis	9	18	15	15
Bacillus cereus	-	-	-	-
Bacillus cereus31	14	20	20	16
Streptococcus faecalis	28	25	35	20
Micrococcus luteus	21	-	-	-
Corynebacterium hoffmonii	27	-	15	-
Corynebacterium xerosis	19	14	24	-

C = Clove oil, K = Kalonji oil, T = Taramira oil, M = diluted methanol (- control), - = no zone of inhibition.

Table 2. Antibacterial activity of *Syzigium aromaticum* (Clove), *Nigella sativa* (Kalonji) and *Eruca sativa Miller* (Taramira) oils and rice bran (Against gram negative bacteria).

Organism	Zones of inhibition (in mm)			
	T	K	C	M
Escherichia coli 5014	22	15	26	-
Escherichia coli	20	-	21	-
Enterobacter aerogenes	10	-	-	-
Klebsiella pneumoniae	20	18	20	-
Salmonella typhi	20	14	21	-
Salmonella para typhi A	15	-	20	-
Salmonella para typhi B	-	-	-	-
Pseudomonas aeruginosa	18	-	18	-
Proteus vulgaris	20	12	-	-

C = Clove oil, K = Kalonji oil, T = Taramira oil, M = diluted methanol (- control), - = no zone of inhibition.

sativa (Kalonji) oils on growing cells of K. *pneumonia* were found to be bactericidal as inhibition of the microbial cells was observed (Figures 4 and 5). Clove oil showed maximum inhibition of K. *pneumoniae* upto 4 h as compared to Kalonji oil.

Chemical analysis of oils

Oils were chemically analyzed to determine their composition as shown in Table 7. It was found that Taramira oil has higher moisture content as compared to other tested oils.

Free fatty acid is defined as the percentage by weight of free acid groups in the oil whereas acid value is defined asthe weight (mg) of KOH required to neutralize free acid groups. Furthermore, Iodine value of Kalonji and Taramira oils was found to be 23.173 and 41.950 ml, respectively.

Table 3. Standard antibiotic activity against gram positive bacteria

Organism	Zones of inhibition (in mm)			
	Ce	CH	A	S
Staphylococcus aureus	38	-	-	28
Staphylococcus epidermidis	-	-	-	27
Bacillus subtilis	25	10	-	29
Bacillus cereus	23	18	11	30
Bacillus cereus31	30	21	13	30
Streptococcus faecalis	30	15	-	17
Micrococcus luteus	40	23	12	40
Corynebacterium hoffmonii	40	-	-	32
Corynebacterium xerosis	47	17	19	40

Ce= Cefamezin, CH= Chloramphenicol, A= Ampicillin, S= Streptomycin, - = no zone of inhibition.

Table 4. Standard antibiotic activity against Gram negative bacteria

Organism	Zones of inhibition (in mm)			
	Ce	CH	A	S
Escherichia coli 5014	40	-	-	26
Escherichia coli	32	-	-	26
Enterobacter aerogenes	-	12	-	22
Klebsiella pneumoniae	-	-	-	-
Salmonella para typhi A	28	-	-	22
Salmonella para typhi B	28	-	-	28
Salmonella typhi	15	-	-	30
Pseudomonas aeruginosa	-	-	-	25
Proteus vulgaris	-	-	-	19

Ce = Cefamezin; CH = Chloramphenicol; A = Ampicillin; S = Streptomycin; - = no zone of inhibition.

DISCUSSION

Increasing antimicrobial resistance is a growing threat to human health and is mainly a consequence of overuse of antibiotics. Administration of antibiotics greatly disrupts the intestinal microflora, there is always a chance that the patient will become colonized by resistant organisms and develop serious infections. Current study was focused to investigate the antibacterial and antifungal potential of natural edible oils that is, *S. aromaticum* (Clove), N. sativa (Kalonji) and *E. sativa* Miller (Taramira) oils in order to treat infectious diseases caused by bacteria and fungi. To the best of our knowledge, limited studies have focused on antimicrobial potential of Taramira oil. In the early experiments, we also used rice bran in order to

Table 5. Antifungal activity of standard antibiotic (Nystatin) and *Syzigium aromaticum* (Clove), *Nigella sativa* (Kalonji) and *Eruca sativa Miller* (Taramira) oils

Organism	Zones of inhibition (in mm)				
	K	C	T	M	N
Penicillium	-	-	20	-	20
T. rubrum	-	-	-	-	16
T. mentagrophyte	-	20	16	-	20
M. canis	-	-	25	-	17
M. gypsum	-	-	34	-	20
Nigrosporum	-	-	40	-	20
Cunninghamella	-	-	24	-	22
A. niger	-	-	20	-	18
A. flavus	-	-	20	-	18

C = Clove oil; K= Kalonji oil; T= Taramira oil; M= Diluted methanol (- control); N= Nystatin (+ control); - = no zone of inhibition.

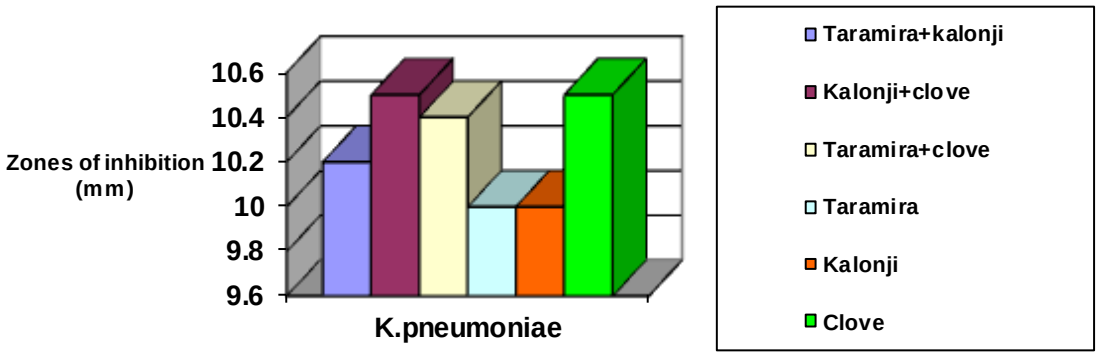

Figure 1. Combined effect of oils (1:1) against *Klebsiella pneumonia*.

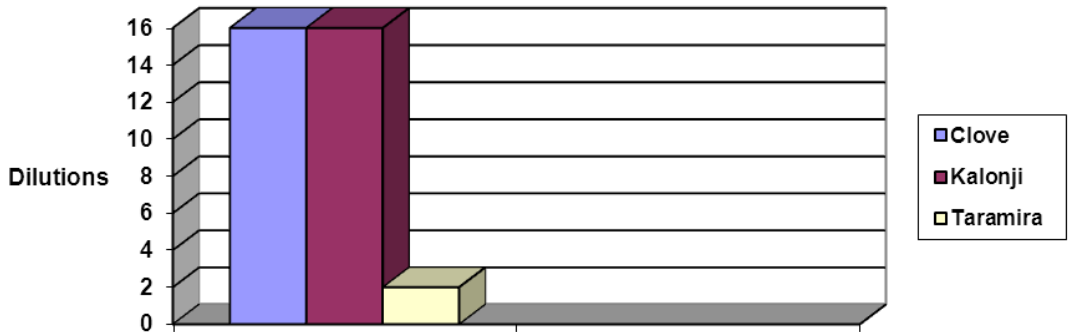

Figure 2. Activity unit (AU/ml) of Clove, Kalonji and Taramira oils against *Klebsiella pneumoniae*).

assess its antimicrobial spectrum but unfortunately we did not get any significant results.

It was found that Gram negative bacteria and fungi were more sensitive to all the tested oils. Bii et al. (2010) reported good activity of methanol extracts of Prunus africana against some bacterial and fungal strains. In our study, K. *pneumonia*, A. *flavus* and *Cunninghamella* were found to be more sensitive microorganisms showing larger zones of inhibition, and mode of action of Clove and Kalonji oils were bactericidal. Klebsiella sp. causes wound, respiratory and catheter related infections while A. *flavus* and *Cunninghamella* are considered as

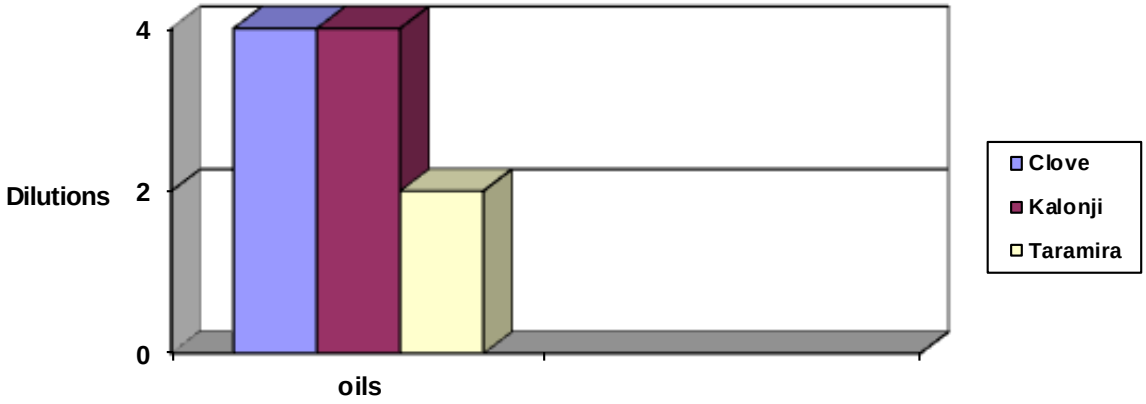

Figure 3. MICs of Clove, Kalonji and Taramira oils (against *Klebsiella pneumoniae*).

Table 6. Activity unit and MICs of Clove, Kalonji and Taramira oils (Against *Klebsiella pneumoniae*).

Oil	**Activity unit** (AU/ml)	MIC
Clove	1:16	1:4
Kalonji	1:16	1:4
Taramira	1:2	1:2

Table 7. Chemical analysis of S*yzigium aromaticum* (Clove), *Nigella sativa* (Kalonji) and *Eruca sativa Miller* (Taramira) oils.

Chemical analysis	Clove	Kalonji	Taramira
Moisture content (%)	9.22	7.79	12.25
Free fatty acids (%)	0.21	0.841	0.168
Acid value (%)	0.418	1.673	0.334
Iodine value (ml)	ND	23.173	41.950

ND= Not done.

pathogenic fungi. Infections caused by E. *coli*, K. *pneumoniae*, *Salmonella typhi* and P. *aeruginosa* may be treated with these oils as they are more active against gram negative bacteria. *P. aeruginosa* is an opportunistic human respiratory pathogen and has high degree of resistance to many antibiotics (Pearson et al., 2000). *P. aeruginosa* has distinct properties which enable the bacteria to colonize and infect Cystic Fibrosis lung (Matsui et al., 2006). One of the study determined antimicrobial sensitivity of Clove oil against some Gram negative bacteria including E. *coli*, *Klebsiella pneumoniae*, *Salmonella paratyphi*, Gram positive bacterium S. *aureus* and a fungus *Candida albicans* where Clove oil showed a broad spectrum activity (Ayoola et al., 2008). Number of studies reported antibacterial activity of *N. sativa* seeds. Their seeds contain tannins, which can be extracted by methanol (Eloff, 1998) and have reported antimicrobial properties (Scalbert, 1991).

Recent work also focused to test these oils for their anti dermatophytic activities as different studies focused antifungal potentials of Clove oils (Soliman and Badea, 2002; Velluti et al., 2003; Feng and Zheng, 2007; Lopez-Malo et al., 2007). Taramira can be used to treat fungal infections and it may be used as anti-dandruff as it exhibits maximum activity against tested fungi.

Drug synergism between known antibiotics and bio-active plant extracts is a novel concept and could be beneficial (synergistic or additive interaction) or deleterious (antagonistic). In this study, no significant results were obtained by combination of oils with each other and with antibiotics. These results were contradictory with the fact that the combination of two agents exhibit significant synergism only if the test organism is resistant to at least one of the agents (Esimone et al., 2006). Adwan et al. (2008) determined additive interactions between antimicrobial agents and plant extracts against five strains of S. *aureus*.

Activity units and MIC values of Clove and Klaonji oils were found to be higher than Taramira oils. These results are in agreement with those found by Udomlak Sukatta et al. (2008) who demonstrated that the MIC values of Clove oil were greater than those obtained from cinnamon oil.

Chemical analysis was done to investigate the overall moisture content, presence of free fatty acids and nature of bonding in the tested oils. The results obtained from chemical analysis revealed that the Taramira oil is more unsaturated than Kalonji oil as iodine value is used to determine the level of unsaturation in oils or fats. The traditional iodine value determination method using the wigs reagent requires CCl_4. We used cyclohexane in place of CCl_4. Wigs reagent is iodine solution in acetic acid which provides iodine monochloride to react with double bonds. Water forces the oil into cyclohexane and the excess iodine monochloride moves into the water,

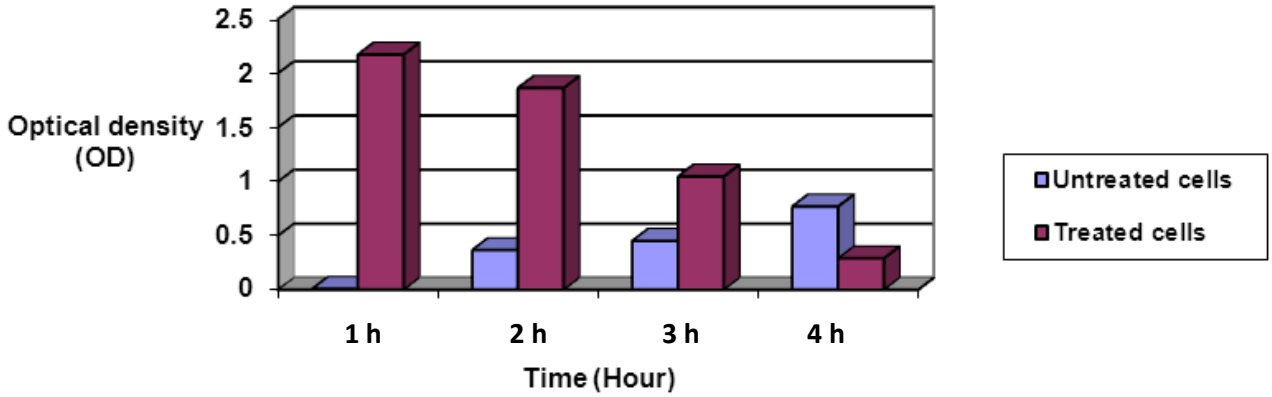

Figure 4. Effect of *Syzigium aromaticum* (Clove) oil on growing cells of *Klebsiella pneumonia.*

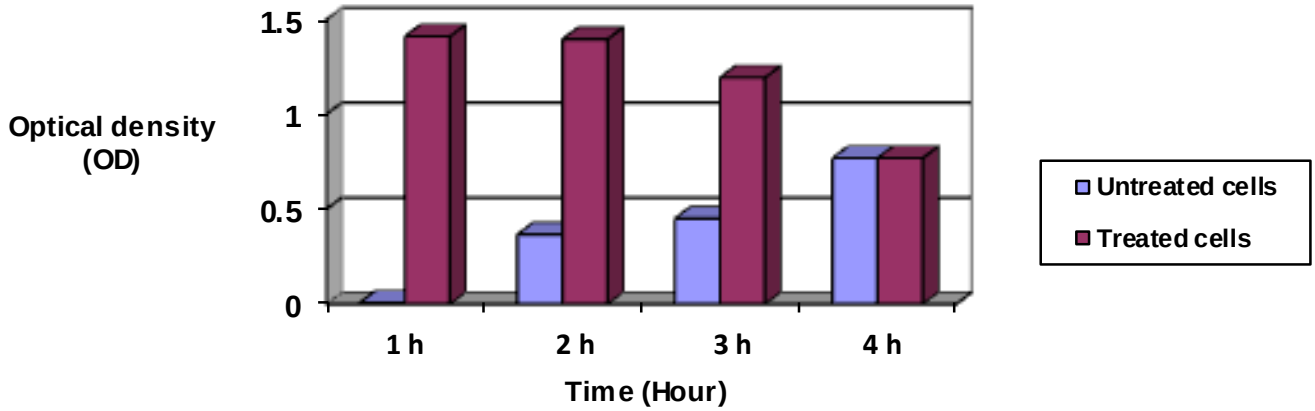

Figure 5. Effect of *Nigella sativa* (Kalonji) oil on growing cells of *Klebsiella pneumonia.*

where it is converted to I2 and can be titrated with water soluble $Na_2S_2O_3$. KI solution converts excess iodine monochloride to free iodine (blue) which can be titrated to a colorless end point with Na2S2O3. As compared to other tested oils Kalonji has high free fatty acid and acid values.

Conclusion

Present work reveals that crude oils of Clove, Kalonji and Taramira possess antimicrobial activity. Infections caused by *P. aeruginosa, Klebsiella* sp., S. *typhi* may be treated with these oils as they are more active against Gram negative bacteria. Taramira can be used to treat fungal infections and it may be used as anti-dandruff as it exhibits maximum activity against tested fungi. Thus we can conclude that these oils can be used as bio control agents to treat bacterial and fungal infections. However, further *in vivo* studies are required to evaluate the potential use of these oils as antimicrobial agents in tropical or oral applications.

Conflict of Interest

None of the authors declared financial and social conflict of interest regarding this study.

ACKNOWLEDGEMENTS

We are thankful to Prof. Dr. Shakeel Ahmed, Department of Microbiology, University of Karachi for his cooperation and support.

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