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Full Length Research Paper

Heavy metals bioremediation potential of *Klebsiella* species isolated from diesel polluted soil

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Heavy metals are transition elements whose presence at mild doses in water bodies is deadly. Most effectively harnessed technique of their remediation is the microbial approach, also known as bio-remediation. This study is hereby aimed to investigate the potential of *Klebsiella* species isolated from diesel polluted soil in bioremediating the heavy metals present in effluent water. A sample of diesel polluted soil was obtained from an automobile repair shop in Ile-Ife, Osun State Nigeria. Bacterial strains from the sample were isolated, characterized and identified by biochemical techniques. Three of the isolates were found to be *Klebsiella* species. Each of the strains and their consortium were administered in a 16-day bioremediation study, into sterile digested effluent water from a stream in Ile-Ife, Osun State, Nigeria. Each of the strains was tolerant to the presence of the heavy metals compared to the consortium, except with Chromium which gave consistency in both administrations. The *Klebsiella* species were very tolerant to Chromium. Cadmium follows suit with copper in better tolerance while there seem much resistance to Nickel. The concentration of Chromium was reduced from 2.718 to 0.046 mg L^{-1}, 0.039, 0.041 and 0.047 mg L^{-1} by *Klebsiella edwardsii*, *Klebsiella oxytoca*, *Klebsiella pneumoniae* and the consortium of the strains, respectively. Cadmium was also reduced from 0.027 to 0.002 mg L^{-1}, 0.001, 0.003 and 0.002 mg L^{-1} by *K.edwardsii*, *K. oxytoca*, *K. pneumoniae* and the consortium of the strains, respectively. The same list of organisms respectively reduced copper from 0.173 to 0.022 mg L^{-1}, 0.025 0.018 and 0.030 mg L^{-1}, and reduced Nickel from 0.103 to 0.019 mg L^{-1}, 0.020, 0.017, and 0.017 mg L^{-1}, respectively. It can be concluded that the trend of each microbe's and the consortium's affinity for the heavy metals uptake is predictably in the trend Cr > Cd > Cu > Ni.

Key words: Bio-remediation, diesel polluted soil, effluent water, tolerant, resistant, heavy metals, *Klebsiella* species.

INTRODUCTION

The main threats to human health from heavy metals are associated with exposure to lead, cadmium, mercury and arsenic amongst others. These metals have been extensively studied and their effects on human health are regularly reviewed by international bodies such as the World Health Organization (WHO). Heavy metals have

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been used by humans or involved in most human activities such as mining operations, metal smelting, foundry works, metal moulding operations, etc. for thousands of years (Järup, 2003).

Although, several adverse health effects of heavy metals have been known for a long time, and exposure to these metals is increasing every day. This is being experienced in some parts of the world, in particular in less developed countries (Järup, 2003). The commonest techniques of removal of heavy metals include chemical precipitation, oxidation or reduction, filtration, ion-exchange, reverse osmosis, membrane technology, evaporation and electrochemical treatment. These techniques become ineffective when the concentrations of heavy metals are less than 100 mg L^{-1} hence, need for the use of microbes for remediation (Ahluwalia and Goyal, 2007).

Indigenous diesel polluted sites are potential sources of heavy metals degraders other than the hydrocarbon itself (Akpoveta and Osakwe, 2010). Diesel, a refined petroleum fraction, is known to contain a known range or limit of heavy metals as part of its constituents. Hence, continual spillage of the diesel on both soil and water bodies, makes the host bodies prone to having accumulating doses of these heavy metals.

Heavy metals are evident constituents of petroleum diesel for heavy duty engines, which constitutes significant content of exhausts' particulate matter (Akpoveta and Osakwe, 2010; Bharathi et al., 2005; Sharma et al., 2005). Amongst the many inadvertent use of the diesel is its spillage at many automobile workshop centres on both the soil and water drainages as well as through pipeline bunkering. Hence, increase in bioavailability of these heavy metals in these host bodies is inevitable.

Due to these activities, heavy metals in diesel oil can be deposited in the soil and enter the human food web thereby constituting risk to the ecosystem, as they tend to bio-accumulate which can be transferred from one food chain to another. Consequently they are discovered in various food chains, where the results are usually detrimental to plants, animals and humans alike (Abdu et al., 2007).

Most of the deleterious cases of heavy metals contamination are treated biologically, based on the safe metabolic pathways peculiar to the degrading agents and the environmental friendliness of the process involved, leaving no pollutants to the risk of the sustainable environment (Mulligan et al., 2001; Gavrilescu, 2004; Rezaee et al., 2005; Elouzi et al., 2012).

Biological treatment is an innovative technology available for heavy metal polluted wastewater. Since microorganisms have developed survival strategies in heavy metal polluted habitats, their different microbial detoxifying mechanisms such as bio-accumulation, bio-transformation, bio-mineralization or biosorption can be applied either *ex situ* or *in situ* to design economical bioremediation processes (Dixit et al., 2015; Lin and Lin, 2005; Malik, 2004). Many are the cases of the heavy metals removal by biosorption with further transformation thereafter (Volesky, 1990).

Uptake of heavy metals into the cellular structures of the microbial agent is dependent on the tolerance of the organism for the particular heavy metal (Mustapha and Halimoon, 2015; Rajendran et al., 2003). According to Dixit et al. (2015), the uptakes of heavy metals in the microorganisms are actively (bio-accumulation) and/or passively (adsorption).

The microbial cell walls, which mainly consist of polysaccharides, lipids and proteins, offer many functional groups that can bind heavy metal ions, and these include carboxylate, hydroxyl, amino and phosphate groups. Among various microbe-mediated methods, the biosorption process seems to be more feasible for large scale application compared to the bio-accumulation process, because microbes will require addition of nutrients for their active uptake of heavy metals, which increases the biological oxygen demand or chemical oxygen demand in the waste.

Several microorganisms have been extensively studied for heavy metal bio-remediation. These include the fungi of genera *Penicillium*, *Aspergillus* and *Rhizopus*; and the bacterial species being *Bacillus* and *Pseudomonas* species (Huang and Huang, 1996, Volesky and Holan, 1995). However, amongst bacterial strains of unique and significant tolerance for heavy metals, which are yet to be researched extensively are the *Klebsiella* species. They are gram-negative, facultative, non-motile, usually encapsulated rod-shaped bacteria belonging to the family Enterobacteriaceae (Niemelä and Väätänen, 1982). These are often found in pristine and hydrocarbon polluted sites, such that they can degrade the heavy metals tolerable to them and the hydrocarbons as well (Kumaran et al., 2011; Nwinyi et al., 2014; Rodrigues et al., 2009).

Klebsiella pneumoniae and *Klebsiella oxytoca* are well-known strains with high tolerance for cadmium and arsenic, respectively (Shakoori et al., 2010, Shamim and Rehman, 2012). This study is hereby aimed to investigate the potential of Klebsiella species isolated from diesel polluted soil in bioremediating the heavy metals present in effluent water obtained from Ile-Ife community, Osun State Nigeria..

MATERIALS AND METHODS

The effluent water bio-remediated for heavy metals, are obtained from the stream in Ile-Ife community, having its source from automobile workshops and filling station. Reagents and salts used were all of analytical grade.

Microbial isolation, characterization and identification

The soil sample was prepared by suspending 0.5 g of the diesel-oil-

polluted soil sample, in 50 ml of sterile distilled water, in a 100 ml conical flask in which some glass chips were inserted. The flask was carefully agitated to obtain a uniform suspension. 0.1 ml of the suspension was taken and added to 9.9 ml of distilled water in test tube A, then 0.1 ml of the suspension in test tube A was taken and added to 9.9 ml of distilled water in test tube B. 1.0 mm of each dilution in tubes A and B was transferred into properly labelled petri dishes, respectively. Culturing was done by making use of 20 ml molten sterile Eosin Methylene Blue (EMB) agar. The culture plate was carefully rotated to mix the cell suspension with the medium. The culture plates were allowed to stand for the EMB agar to set. They were incubated at 35°C for 48 h. Colonial characteristics of the mixed culture obtained were observed. The differential colonies were isolated by sub-culturing into nutrient agar slants and labelled accordingly for use. The 18 h cultures were gram stained, to study the morphological characteristics of the cultures (i.e. cell shape, cell arrangement and gram reaction) and to ensure their purification.

Gram staining process

According to Gunasekaran (2007), microscope slides were soaked in chromic acid, washed properly and cleaned with cotton wool soaked in ethanol. The slides were labelled. A sterile inoculating loop was used to transfer a loopful of the broth culture and spread on a slide to obtain a thin film (smear). The smear was heat fixed, flooded with crystal violet for 60 s and rinsed off with gram iodine solution for absorption to take place. The iodine was left to act for 1min. Decolourization of the smears was done by adding 95% ethanol for 60 s and rinsed off with tap water. The slides were counterstained with 1% carbolfuschsin for 1 min after which, they were rinsed with tap water to stop the reaction and blot dried. The prepared slides were then viewed under a microscope and the results recorded.

Biochemical characterization of bacterial isolates

The bacterial isolates were subjected to various biochemical procedures for characterization, according to Collins (1989). These are:

1. Triple sugar ion test
2. Sulphide-indole-motility test
3. Catalase test
4. Citrate utilization test
5. Methyl red test
6. Voges-proskauer test
7. Nitrate reduction
8. Oxidation fermentation test
9. Oxidase test
10. Sugar fermentation test

Heavy metal resistance test

Molten sterile nutrient agar containing the heavy metals (Cu^{2+}, Ni^{2+}, Cd^{2+} and Cr^{3+}) were prepared in varying concentrations and a loopful of each bacteria isolate was collected and streaked on the surface of the heavy metal enriched medium. This procedure was carried out for each of the isolated microorganisms. Thereafter, the plates were incubated at 37°C for 24 h and monitored for growth.

Digestion of water samples for bioremediation

A digestion process was carried out on 10 ml of effluent water sample. 10 mm of aqua regia and 1 ml of perchloric acid were added into the measured water sample, in a conical flask. The mixture was then placed in a water bath at 80°C.

After total digestion and subsequent cooling, the resulting solution was diluted to 50 ml mark of a 50 mm standard volumetric flask with de-ionized water and analysed for heavy metals using atomic absorption spectrophotometer, AANALYST400 (Ogoyi et al., 2011). The heavy metals analysed were Cu, Ni, Cr and Cd.

Inoculum preparation

A suspension of standardized bacterial-saline medium was constituted for each isolate and the consortium. Each of the isolates was grown in nutrient broth for 18 hours, and the bacterial cells pelleted by centrifugation at 4000 rpm for 15 min at 4^{o}C using Eppendorf Centrifuge 5804R, New Brunswick, New Jersey, USA. The pellet was then rinsed twice and resuspended using 0.85 % NaCl to give an OD_{600nm} of $\geq$0.5. The consortium culture was prepared by mixing each of the single isolates in equal portion and top up with 0.85 % NaCl to give a final reading of OD_{600nm} $\geq$0.5, corresponding to approximately 1×10^7 colony-forming units per ml as determined by spread plate method. This was used as the starting culture in all subsequent experiments (Basha and Rajaganesh, 2014; Wong et al., 2015).

Bio-remediation of heavy metals

250 mm of Bushnell-haas medium was prepared and 10 ml was distributed into four conical flasks, each of which contains 10 ml of diesel-polluted-water. Sterilization by autoclaving was done at 121°C, 15 psi for 15 min.

The sterile digested water sample was aseptically inoculated with 5 ml of the inoculum, of pure and mixed cultures of *Klebsiella* species. The flasks were incubated on a New Brunswick Gyratory shaker for 16 days. Samples were withdrawn for four days interval for analysis, using the atomic absorption spectrophotometer.

RESULTS AND DISCUSSION

Isolation, gram staining, biochemical characterization of isolates and heavy metals resistance tests

Four bacterial isolates were obtained from the diesel-oil-polluted soil with each of them, being given an isolate code. Colonial characteristics of the individual isolates showed that Isolate A was large, regular, slightly raised and violet at the centre on EMB agar. Isolate C was large, regular, raised and sticky. Isolate E was irregular, spread, slightly raised, mucoid and sticky while organism F was seen to be large, regular, flat, rough and yellowish in colour. Gram staining result showed long gram negative bacilli chains.

Table 1 shows the results of the morphological characterization of the isolates. Based on the colonial, morphological and biochemical characteristics, the isolates were identified as *Pseudomonas cepacia* (isolate A), *Klebsiella edwardsii* (isolate C), *Klebsiella oxytoca* (isolate E) and *Klebsiella pneumonia*e (isolate F). This result was in agreement with the work of Adeyemo et al. (2013) in which, *Klebsiella* species was isolated from a diesel contaminated soil. Isolates C, E, and F showed better growth on the sterile EMB agar than isolate A. *P.*

Table 1. Biochemical characterisation of some bacteria obtained from a diesel-oil-polluted soil.

Code	C.S	G.R	C.T	OX	TSI	SIM	CT	MR	VP	GLU	S.F MALT	MANN	SUC	LACT	O.F	N.R	Organisms
A	MLR	—	+	+	N N N	— — —	—	—	—	—	—	—	—	—	Ox	+	*Pseudomonas cepacia*
C	MLR	—	+	—	Y N N	— — —	+	+	+	Y	Y	Y	Y	Y	F	+	*Klebsiella edwardsii*
E	LR	—	+	—	Y N N	— — —	—	+	+	YG	YG	YG	YG	YG	F	+	*Klebsiella oxytoca*
F	MLR	—	+	—	Y N N	— — —	+	+	—	Y	NC	NC	NC	NC	F	+	*Klebsiella pneumoniae*

CS: Cell shape; GR: Gram reaction; CT: Catalase; OX: Oxidase; TSI: Triple sugar ion reaction; SIM: Sulphide indole motility; CTU: Citrate utilization; MR: Methyl-red; VP: Voges proskaur; GLU: Glucose; MALT: Maltose; MANN: Mannitol; SUC: Sucrose; LACT: Lactose; OF: Oxidation-fermentation; SF: Sugar fermentation; NR: Nitrate reduction; Y: Acid production only; YG: Acid and GAS production; N: No change; F: Fermentative; OX: Oxidative; SR: Short rod; MLR: Medium long rod; LR: Long rod.

Table 2. Data for bioremediation of heavy metals in diesel-polluted-water using a pure culture of *Klebsiella edwardsii.*

Heavy metal	Initial Concentration (mg/L)	Day 4 (percentage reduction %)	Day 8 (percentage reduction %)	Day 12 (percentage reduction %)	Day 16 (percentage reduction %)
Copper	0.173	0.040 (76.9)	0.032 (81.5)	0.027 (84.4)	0.022 (87.3)
Nickel	0.103	0.057 (44.7)	0.040 (61.2)	0.030 (70.9)	0.019 (81.6)
Chromium	2.718	1.758 (35.3)	1.263 (53.5)	0.638 (76.5)	0.046 (98.3)
Cadmium	0.027	0.004 (85.2)	0.003 (88.9)	0.002 (92.6)	0.002 (92.6)

cepacia, K. edwardsii, K. oxytoca and K. pneumoniae were resistant to Ni and Cd which is corroborated by the work of Kumaran et al. (2011).

Bio-remediation of heavy metals

Using Klebsiella edwardsii

The obtained results of the determined concentrations of each heavy metals analysed are as stated in Table 2. The Table 2 further shows the reduction in heavy metal concentration at intervals of 4 days. The results indicate that, *K. edwardsii* has an increasing tolerance of all the metals but most for Chromium through the 16 days of the study.

Within the first 4 days, tolerance for four metals was least in Chromium perhaps due to the higher concentration of Chromium in the medium compared to the sum of the concentrations of others. The tolerance grew more steadily for Chromium than for each of the other 3 metals. This relation is depicted in Figure 1.

Using Klebsiella oxytoca

Similarly, *K. oxytoca* gave results as contained in Table 3. The results in Table 3 show that the tolerance of *K. oxytoca* after the first 4 days was least for Chromium among the metals but most after the complete 16 days.

The strain showed a progressive affinity for the metals in the order of Cr>Ni>Cu>Cd. The result also shows that *K. oxytoca* significantly reduced cadmium just within the first 4 days. The trend of the reduction in concentration is also depicted in Figure 2.

Using Klebsiella pneumonia

K. pneumoniae showed high tolerance for selected heavy metals in such a way that, it was most for Chromium but least with Cadmium after the first 4 days. Table 4 and Figure 3 reveal the trend of degradation.

Using the consortium of the Klebsiella species

The potency of the consortium, except Chromium in the bioremediation was observed to be relatively low compared to the significant reduction by the use of each *Klebsiella* species

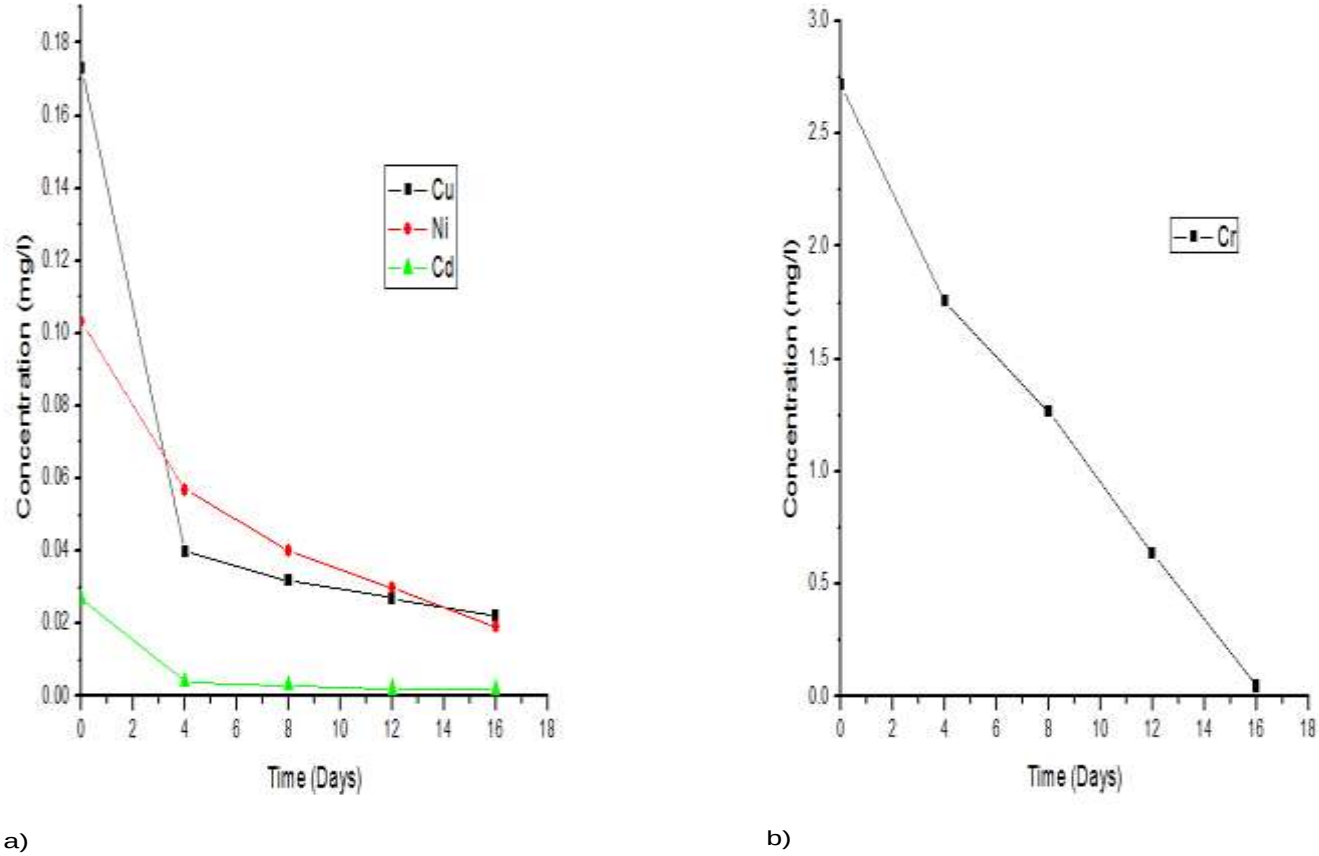

Figure 1. Plot of reduction in concentration of the heavy metals by *K. edwardsii* ((a) Graph of reduction in concentration of copper, nickel, and cadmium with time using *K. edwardsii* as bioremediating agent; (b) Graph of reduction in concentration of chromium with time using *K. edwardsii* as bioremediating agent).

Table 3. Data for bioremediation of heavy metals in diesel-polluted-water using a pure culture of *Klebsiella oxytoca.*

Heavy metal	Initial concentration (mg/L)	Day 4 (%)	Day 8 (%)	Day 12 (%)	Day 16 (%)
Copper	0.173	0.055 (68.2)	0.053 (69.4)	0.038 (78.0)	0.025 (85.5)
Nickel	0.103	0.047 (54.4)	0.042 (59.2)	0.030 (70.9)	0.020 (80.6)
Chromium	2.718	1.667 (38.7)	1.375 (49.4)	0.638 (76.5)	0.039 (98.6)
Cadmium	0.027	0.004 (85.2)	0.004 (85.2)	0.003 (88.9)	0.001 (96.3)

isolates as seen in Tables 2 to 4 and Figures 1 to 3.

The results are contained in Table 5 and Figure 4. It is seen in each that, the tolerance of the strains dropped with the individual strain's interference on the metabolic paths. This implies that the interaction of the metals or metal ions on their sorption properties by the strains of study reduces the remediation potential of the organisms. However, the use of the mixed strains proved more tolerant for Chromium as higher reduction in its concentration was obtained across the first 12 days of study before the obvious desorption. The affinity for Cadmium was in line with the result of Shamim and Rehman (2012).

All the gram negative *Klebsiella species* isolates were tolerant to the heavy metals, though with variations. The isolates were capable of reducing the selected heavy metals' concentration unlike the known facts about gram positive and a few gram negative bacterial isolates according to Lima e Silva et al. (2012). The sensitivity of each of the isolates and their consortium was greatest for chromium..

Conclusion

A few heavy metals tolerant- and degrading-bacteria exist

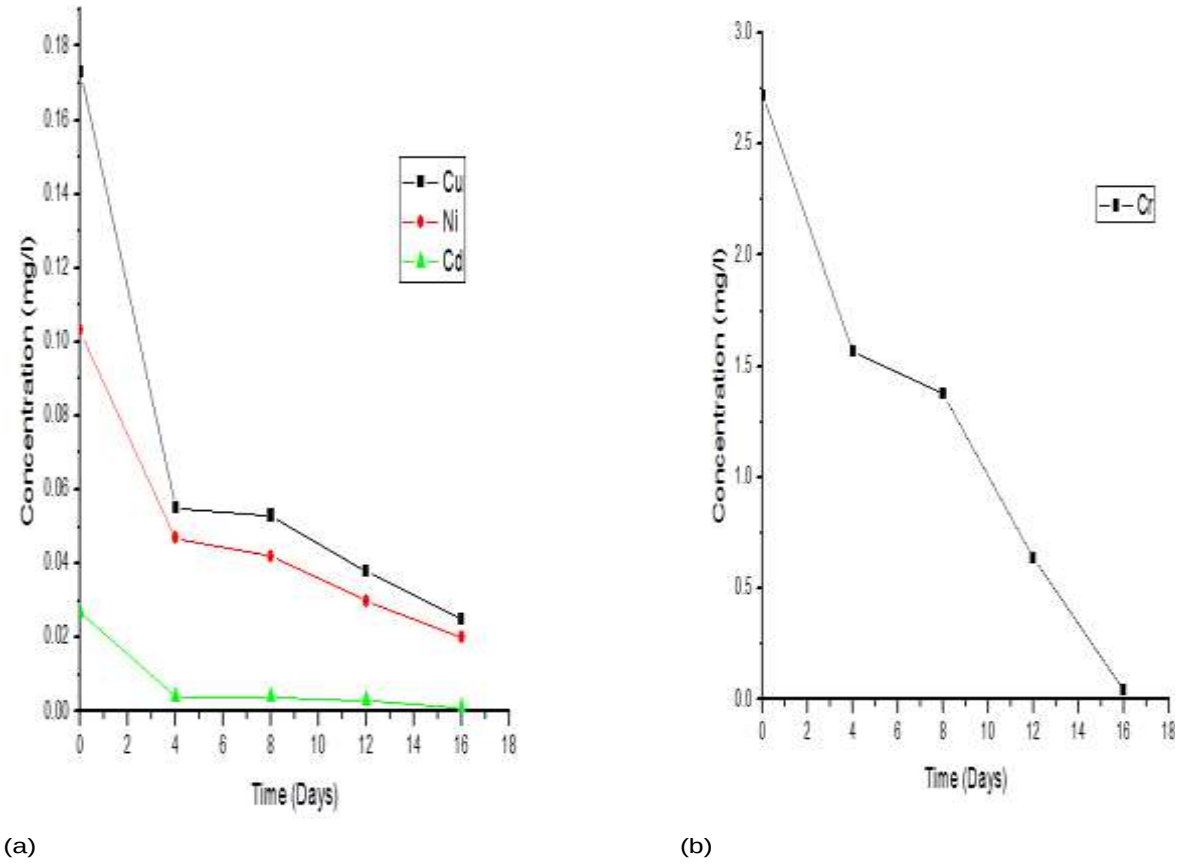

Figure 2. Plot of reduction in concentration of the heavy metals by *K. oxytoca* ((a) Plot of reduction in concentration of copper, nickel, and cadmium with time using *K. oxytoca* as bioremediating agent; Plot of reduction in concentration of copper, nickel, and cadmium with time using *K. oxytoca* as bioremediating agent).

Table 4. Data for bioremediation of heavy metals in diesel-polluted-water using a pure culture of *Klebsiella pneumonia.*

Heavy metal	Initial concentration (mg/L)	Day 4 (%)	Day 8 (%)	Day 12 (%)	Day 16 (%)
Copper	0.173	0.035 (79.8)	0030 (82.7)	0.024 (86.1)	0.018 (89.6)
Nickel	0.103	0.039 (62.1)	0.037 (64.0)	0.026 (74.8)	0.017 (83.5)
Chromium	2.718	1.504 (44.7)	0.569 (79.1)	0.401 (85.2)	0.041 (85.2)
Cadmium	0.027	0.004 (85.2)	0.003 (88.9)	0.003 (88.9)	0.003 (88.9)

Table 5. Data for bioremediation of heavy metals in diesel-polluted-water using a mixed culture of *Klebsiella edwardsii, Klebsiella oxytoca and Klebsiella pneumoiae.*

Heavy metal	Initial concentration (mg/L)	Day 4 (%)	Day 8 (%)	Day 12(%)	Day 16 (%)
Copper	0.173	0.036 (79.2)	0040 (76.9)	0.031 (82.1)	0.030 (82.7)
Nickel	0.103	0.037 (64.1)	0.037 (64.1)	0.025 (75.7)	0.017 (83.5)
Chromium	2.718	1.481 (45.5)	0.440 (83.8)	0.035 (98.7)	0.047 (98.3)
Cadmium	0.027	0.006 (77.8)	0.005 (81.5)	0.004 (85.2)	0.002 (92.6)

in the niche of soil collection. The pure isolates were *P. cepacia*, *K. edwardsii*, *K. oxytoca* and *K. pneumoniae*.

The trend of each microbe's and the consortium's affinity for the heavy metals uptake is, predictably in the trend Cr

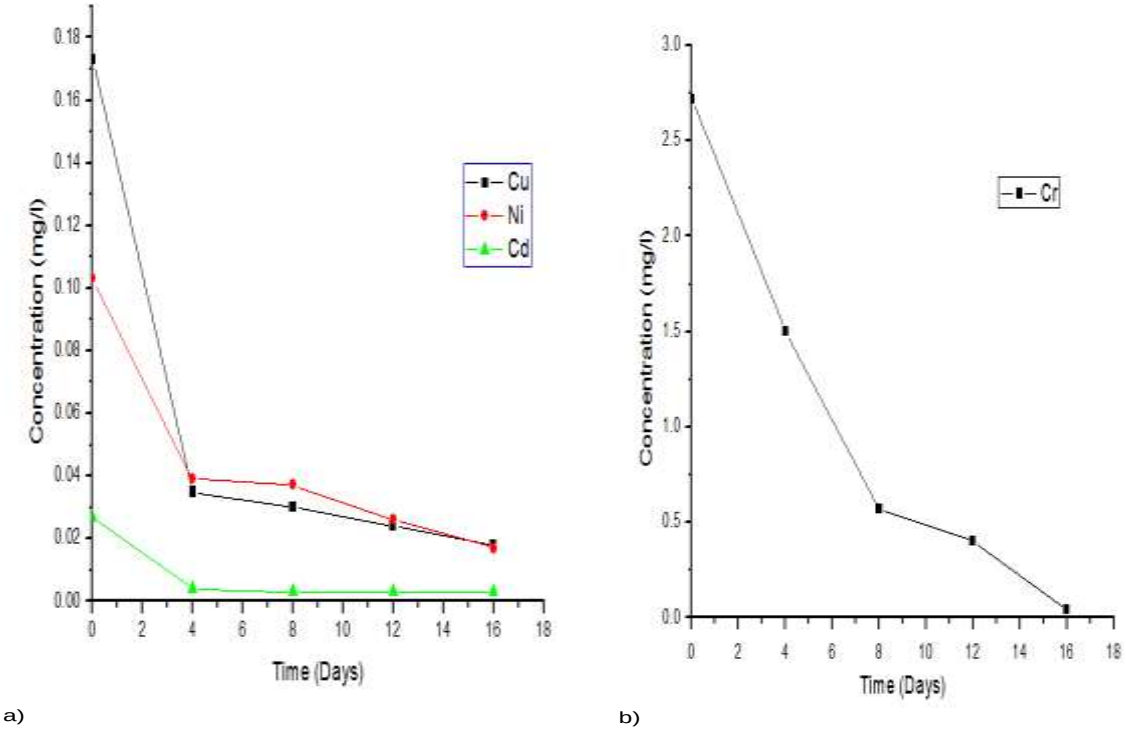

Figure 3. Plot of reduction in concentration of the heavy metals by *K. pneumonia* ((a) Plot of reduction in concentration of Copper, Nickel, and Cadmium with time using *K. pneumoniae* as bioremediating agent; (b) Plot of reduction in concentration of chromium with time using *K. pneumoniae* as bioremediating agent).

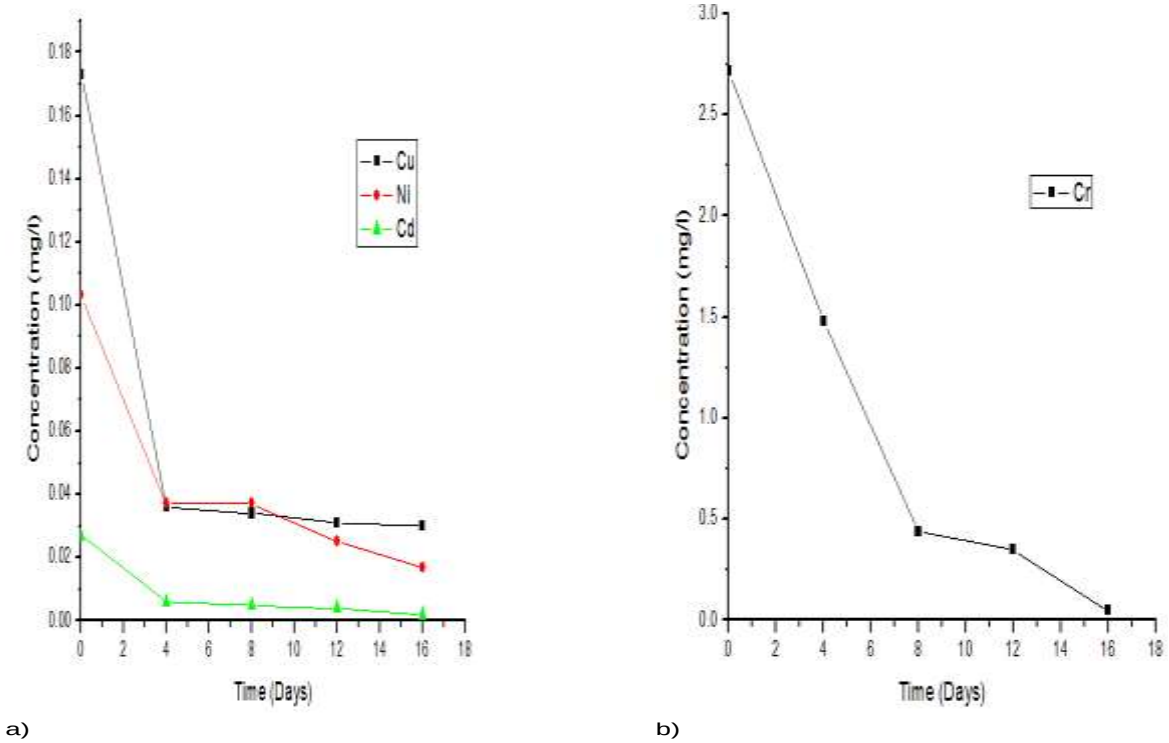

Figure 4. Plot of reduction in concentration of the heavy metals by mixed culture of *K. edwardsii, K. oxytoca* and *K.pneumonia* ((a) Plot of reduction in concentration of cadmium, copper and nickel with time using a mixed culture of *K. edwardsii, K. oxytoca* and *K. pneumonia*; (b) Plot of reduction in chromium with time using a mixed culture of *K. edwardsii, K. oxytoca* and *K. pneumonia.*

> Cd > Cu > Ni.

CONFLICT OF INTERESTS

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

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Full Length Research Paper

Optimized production of lipase from *Bacillus subtilis* PCSIRNL-39

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Lipases catalyze the hydrolysis of long chain triglycerides. Microbial lipases are receiving much attention because of their industrial potential in the chemical, pharmaceutical, medical, cosmetic, biosurfactant synthesis, leather industries, mutation, agrochemicals and paper manufacturing industries. This article presents the isolation of maximum lipase producing bacteria and the optimization of different conditions for the maximum production of lipase. *Bacillus subtilis* PCSIRNL-39 shows maximum production of lipase at 45°C with pH 7 using nitrogen source peptone and carbon source sucrose. *B. subtilis* PCSIRNL-39 showed the best production at 5% inoculum size, while Ca^{2+} and Mg^{2+} were found best stimulator for enzyme production during the study. Tween 20 and 80 enhanced better lipase production than other surfactant. The kinetic parameters of V_{max} and K_m for the lipase were measured to be 101 µM/min.mL and 7.6 mg, respectively.

Key words: *Bacillus subtilis,* microbial lipase, production, optimization.

INTRODUCTION

Lipases are enzymes that catalyze lipids into fats and glycerols. This reaction acts on oil and water interface (Reis et al., 2008). Lipases are involved in certain reactions like aminolysis in organic solvents, tran-sesterification and esterification. Lipases can be obtained from animals, plants and microbes.

The lipase can be produced from microorganisms through the process of fermentation. According to Vakhlu and Kour (2006), lipases are also extracted from plants and animals. Lipases achieved from microbial sources are more stable than that extracted from plants and animals. Bacteria like *Pseudomonas* spp.,

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Staphylococcus spp., *Chromobacterium* spp*., Alcaligeannes* spp*., Achramobacter* spp*.* and *Bacillus* are commercially used for lipase production.

Bacterial lipases can be obtained by submerged fermentation (SmF) as well as solid-state fermentation (SSF). Due to easy production along with inexpensive techniques of fermentation and low energy consumption, the lipases obtained from microbes (fungi, yeast and bacteria) are given preference for industrial application. However, SSF is the most appropriate process due to its various benefits and bioconversion parameters. Lipase production by SSF using olive oil cake and sugar cane bagasse are very cost effective (Cordova et al., 1998) and in this case, oil cakes show best production (Ramachandran et al., 2007).

The most useful lipase producer genus used in industry is *Bacillus (*Jaeger et al., 1994). They are gram positive endospore formers which produce large variety of enzymes, and are considered as strong extra cellular lipase producer. They demonstrate the ability to grow in various ranges of temperature, pH and salt concentration. Due to its stability, the lipases have immense industrial importance.

Recently microbial lipases are used as biosensor. The principle of this biosensor is using lipases to produce glycerol from triacylglycerol, measuring the release of glycerol and quantifying the non-esterified fatty acids. This principle is used to diagnose cardiovascular disorders.

Lipases were produced from locally isolated *Bacillus* species, by using different agro-industrial wastes through solid state fermentation (Mazhar et al., 2016b). This study was designed to investigate physico-chemical parameters for optimized lipase production in cost free medium.

MATERIAL AND METHODS

Microorganism and media

Microorganism culture PCSIRNL-39, used in the study was obtained from Molecular Biology Laboratory at FBRC, LLC Lahore, Pakistan. PCSIRNL-39 was previously isolated from soil and biochemically characterized as *Bacillus* spp. Production medium used for submerged fermentation, had glucose 5 g/l, peptone 5 g/l, yeast extract 5 g/l, olive oil 5%, $MgSO_4.7H_2O$ 0.5 g/l and NaCl 3 gm/l (Mazhar et al., 2016 a,b).

Genotypic characterization

Preparation of genomic DNA

For the purpose of preparing genomic DNA, Ausubel et al. (1994) method was slightly modified. Single colony from pure culture of PCSIRNL-39 was inoculated into LB broth and grown for 24 h at 37°C in water bath shaker.

Overnight grown culture was centrifuged to pellet the cells for 5 min at 8000 rpm. After discarding, the supernatant pellet was resuspended in 567 µl of 1xTE buffer. The addition of 3 µl of 20 mg/ml proteinase K and 30 µl of 10% SDS was made afterwards. The sample was incubated at 37°C for 1 h.

Thorough mixing in Eppendorf tubes, 100 µl of CTAB/NaCl solution (0.7 M NaCl, 10% N-acetyl-N, N, N,-trimethyl ammonium bromide) was added. Incubation was performed at 65°C for 10 min after mixing. One equal volume of chloroform/isoamyl (24:1) was used to perform the chloroform extraction.

Centrifugation of samples started at 10,000 rpm for 5 min after adding equal volume of chloroform/isoamyl. Chloroform extraction was repeated after transferring the aqueous phase into a new Eppendorf tube. 0.6 volume of isopropanol was added in order to obtain the nucleic acid precipitates. Genomic DNA was pelleted down by centrifugation at 10,000 rpm for 10 min. Pellet was washed with 500 µl 70% ethanol, dried at 37°C for 10 min and dissolved in 200 µl of TE buffer.

Amplification of 16S rDNA region

Amplification of 16S rDNA region was performed through polymerase chain reaction (PCR). PCR mix (24 µl) was prepared by adding $MgCl_2$ (25 mM) 1.5 µl, forward and reverse primer (100 µM) each 0.5 µl, sterile deionized water 14.7 µl, dNTP mix (10 mM each) 4 µl, *Taq* buffer (10X) 2.5 µl, *Taq* DNA Polymerase (5 U/µl) 0.3 µl in 0.2 ml PCR tube and 1 µl of genomic DNA was transferred.

PCR sample was centrifuged for 2 to 3 s after mixing gently. All steps were performed on ice and the tubes were placed into Mini Cycler PCR System. The following amplification procedure was adopted.

Step 1: Initial Denaturation: 94°C for 5 min
Step 2: Denaturation: 94°C for 1 min
Step 3: Annealing: 52°C for 1 min
Step 4: Elongation: 72°C for 1 min
Step 5: Extension: 72°C for 10 min final

The following DNA primers were used in this experiment:

Forward primer: 5'-AAACTYAAAKGAATTGACGG-3
Reverse primer: 5'-ACGGGCGGTGTGTRC-3'

Y = C/T; K = G/T; R = A/G

The forward primer was complementary to the upstream of 16S rDNA and the reverse was complementary to the upstream sequences of 23S rRNA gene sequences.

Electrophoresis of amplified 16S rDNA fragments

Agarose gel (0.8%) was used to check the amplification products. 0.8 g of agarose was boiled in 1x TAE buffer (100 ml). This solution was cooled to 40°C and 5 µl of ethidium bromide solution (10 mg/ml) was added. The combs were placed in gel casting stand after pouring the agarose solution to it.

When the gel was set after 20 min, combs were removed and the casting tray was then placed into tank. 1x TAE was added to gel until the surface of the gel was raised by 2 to 3 mm. 1 µl of 6x gel loading buffer was missed with 5 µl of the PCR product. Starting from the second well, the samples were loaded into the agarose gel wells, 3 µl of DNA molecular weight marker was loaded in first well afterward.

Electrophoresis was performed at 100 V for 2.5 h and the PCR products were observed on UV illuminator. Gene clean kit was used to purify the PCR product. Dideoxy chain termination method was used to sequence both strands of PCR product. Blast program was used to make comparison between 16 rRNA gene sequence with NCBI/EZtaxon/Ribosomal Database Project (RPD)/ EMBL nucleotide sequence databases.

Parameters for optimization of lipase production

Different parameters were used to optimize the lipase production such as pH of the medium, incubation time, substrate selection and effect of moisture content of the substrate. Lipase activity was measured according the procedure of Mazhar et al. (2016a).

Effect of pH

Different pH level of the production medium was set ranging from 4.0 to 9.0, using 0.1 N NaOH and 0.1 N HCl. The medium was autoclaved at 15 lbs pressure and 121°C for 20 min.

Effect of temperature

Erlenmeyer flasks (250 ml) were filled with 50 ml of selected production medium and sterilized. The flasks were incubated at 20, 25, 30, 35, 40, 45, 50, 55 and 60°C after inoculation for 72 h.

Optimization of inoculum size

Inoculum size was optimized by inoculating the production broth, using 24 h seed culture of varying size 4, 5, 6, 7, 8, 9, and 10% (v/v). Estimation of growth in batch culture and lipase activity was made.

Different carbon source

For determination of the effect of carbon sources (5 g/l) on growth of lipase *Bacillus* sp., sucrose, glucose, maltose, starch, fructose, lactose and mannitol were used as basal carbon sources. Basal medium was used for assaying and determination of lipase activity.

Different nitrogen sources

Different organic nitrogen sources were also used for optimization. Ammonium sulphate, tryptone, yeast extracts, beef extracts soya bean meal and different combination of these sources were used as nitrogen sources.

Incubation period

Bacillus sp. was grown in agro industrial waste, containing salt media at 45°C in an incubator. It was assayed for lipase activity after various incubation times, that is, from 24 to120 h.

Effect of surfactant on lipase production

The effect of each of the selected surfactant Sodium dodecyl sulphate, viz., Tween-20, Triton X-100 and Tween-80 was separately evaluated, by incorporating (1%; v/v) the surfactant to the production broth which was inoculated with 5% (v/v) inoculums.

Effect of metal ions

Different metal ions such as 1 mM of $CaCl_2$, HCl, $MgSO_4$, NaCl, $MnSO_4$, $ZnSO_4$, Fe_2SO_4, K_2PO_4 and $CuSO_4$ were supplement separately, in the medium. Optimal conditions were used for incubation, and determination of lipase production was done.

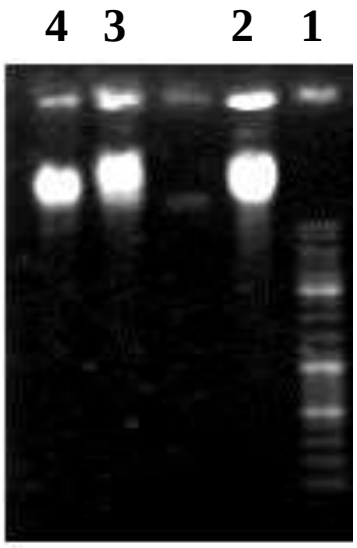

Figure 1. Isolated genomic DNA of bacterial strains: Line 1: DNA marker Ferment as Cat # SM0331; Line 3: DNA of PCSIRNL-39.

Cumulative effect of optimized components

The optimized broth containing 2.5% mustard oil cake and 2.5 (%; w/v) bagasse, in minimal media was calibrated to a final pH of 7.0, to determine cumulative effect of all the selected components on lipase production by *Bacillus subtilis* after 72 h at 45°C.

RESULTS

Genotypic characterization

The genomic DNA of PCSIRNL-39 was isolated as shown in Figure 1. Amplification of 16S rRNA region mostly yielded one distinct DNA fragment, of approximately 500 bp in length (Figure 2).

The amplification product was cleaned with the help of gene clean kit. The size and concentration of purified gene product was determined on agarose gel (Figure 3). Sequencing of the PCR product was preceded by the use of CAMB (Centre for Applied Molecular Biology) sequencing facility.

The sequences obtained were compared with the GenBank database, using the BLASTN tool. PCSIRNL-39 showed 99% homology with published sequences of *B. subtilis*. Sequence for the distinct rDNA fragment was submitted to Gen Bank and accession number, assigned as KT374117.

Optimization of parameters

Effect of temperature

The effect of the incubation temperature on the

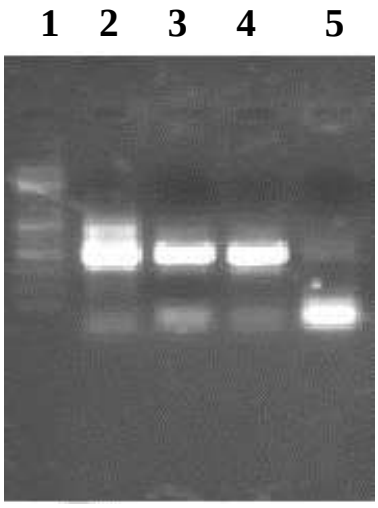

Figure 2. Amplification products of 16S rDNA region of isolated strains: Line 1: DNA Marker Fermentas Cat # SM0331; Line 2, 3, 4: Amplification product 500 bp; Line 5: Control.

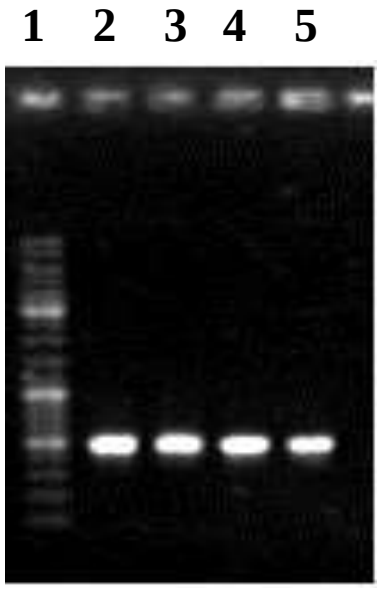

Figure 3. Gene clean product after amplification Line 1: DNA Marker;Fermentas Cat # SM0331; Line 2: PCSIRNL-39.

production of extracellular lipases by *B. subtilis* was investigated. Lipase activity at different incubation temperature such as 25, 30, 35, 40, 45, 50 and 55°C were observed. The maximum lipase production for PCSIRNL-39 was observed at 45°C, 47.50 U/ml. A sharp decrease in lipase production was, however, observed at 50 to 55°C, as shown in Figure 4.

Effect of pH

The effect of pH on the production of extracellular lipase by *B. subtilis* was observed. Lipase activity at different pH such as 4, 5, 6, 7, 8 and 9 were determined. Results inferred that, neutral pH favored the production of extracellular lipase (Figure 5).

Effect of inoculum size

Figure 6 showed that at the inoculum size of 5% the lipase activity was 80.63 U/ml; further increase will cause lipase production to decrease at a high speed.

Effect of carbon source

Starch, glucose, fructose, sucrose, lactose, maltose and manitol were used as carbon source. PCSIRNL-39 showed best results with sucrose and fructose 40.67 and 31.70 U/ml, respectively (Figure 7).

Effect of nitrogen source

The bacterial strain PCSIRNL-39 expressed best lipase production with peptone and yeast extract 50.70 and 45.87 U/ml, respectively (Figure 8). In the presence of inorganic nitrogen [$(NH_4)_2SO_4$, NH_4Cl, and NH_4NO_3), the best lipase production was observed with NH_4NO_3 (45 U/ml). The bacterial strain PCSIRNL-39 showed the least lipase activity, however, with trypton (16.37 U/ml) among all nitrogen sources investigated.

Effect of incubation time

After inoculation, the fermentation media was incubated for different time periods like 24, 48, 72, 96 and 120 h. Experiment revealed that best incubation time for lipase production was 72 h from bacterial strain PCSIRNL 39 used in this experiment (Figure 9).

Effect of metal ions

As reported from this study on microbial lipases, concentration as low as 1 mM of some metal ions, can affect the production of lipase by *Bacillus* sp. Effect of various metals (Mg^2, Na^+, Mn^{2+}, Zn^{2+}, Fe^{2+}, Ag^{2+}, Co^{2+}, Cu^{2+}, Ca^{2+} and K^+) were determined.

In Figure 10, PCSIRNL-39 showed maximum lipase production with Mg^{2+} and Ca^{2+} while Ag^{2+} and Cu^{2+} acted as inhibitors for lipase production.

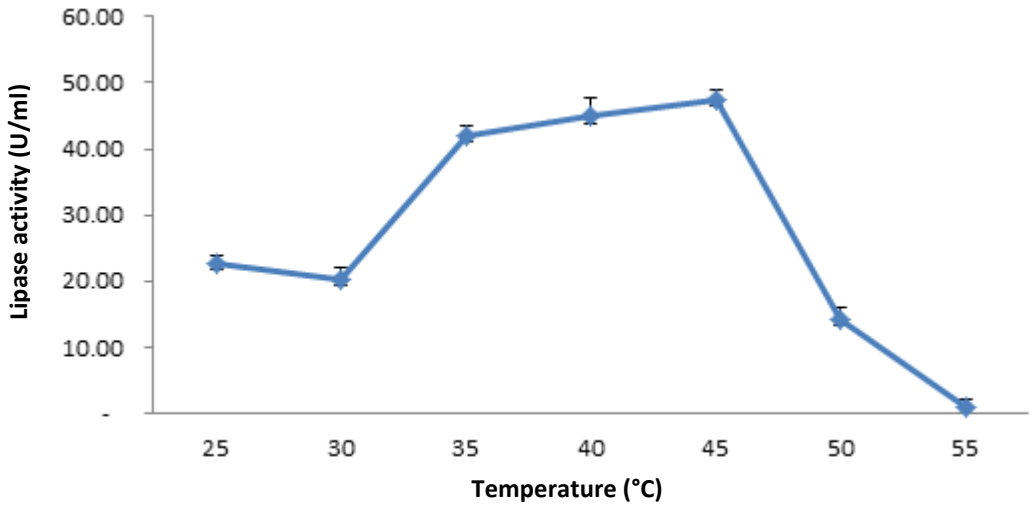

Figure 4. Optimization of temperature for lipase production.

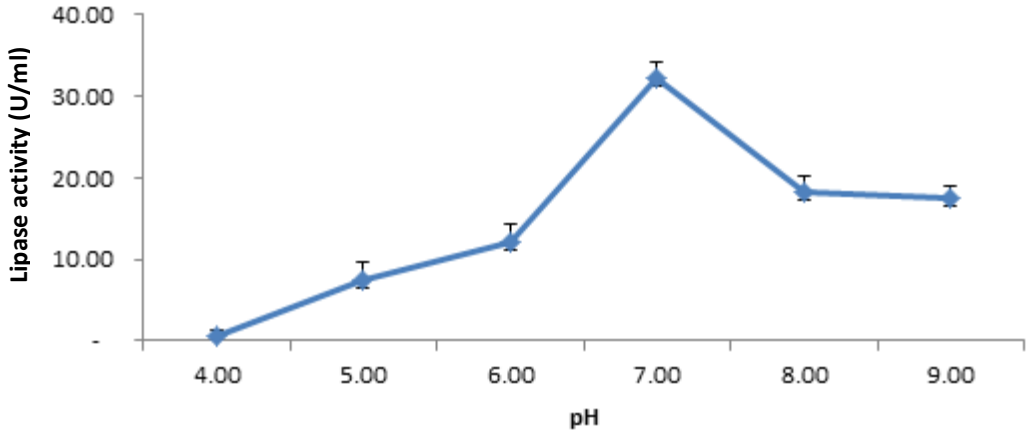

Figure 5. Optimization of pH for lipase production.

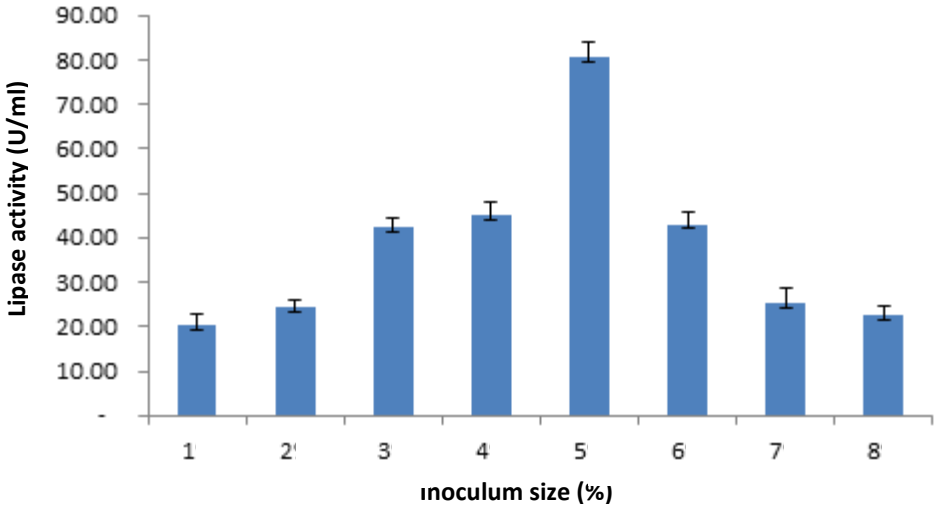

Figure 6. Optimization of inoculum size for lipase production.

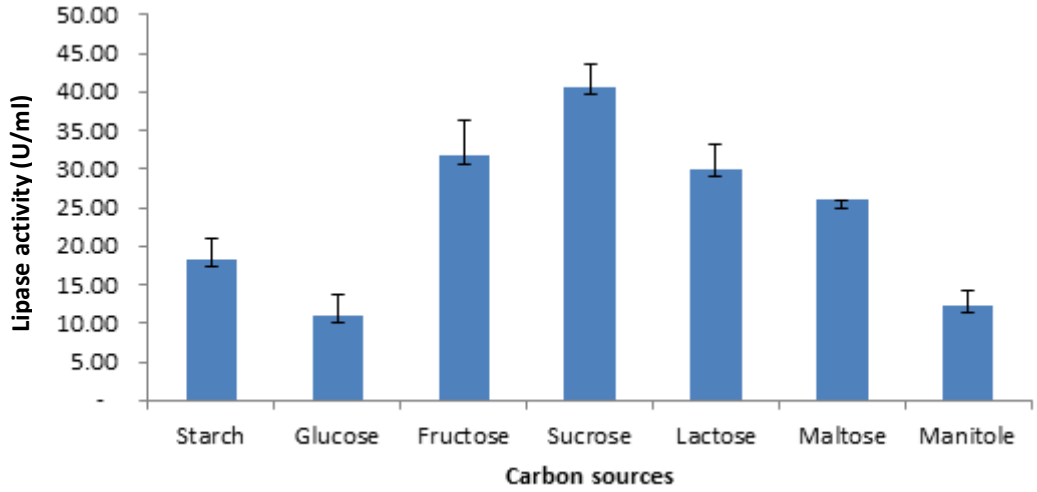

Figure 7. Effect of different carbon sources on lipase production.

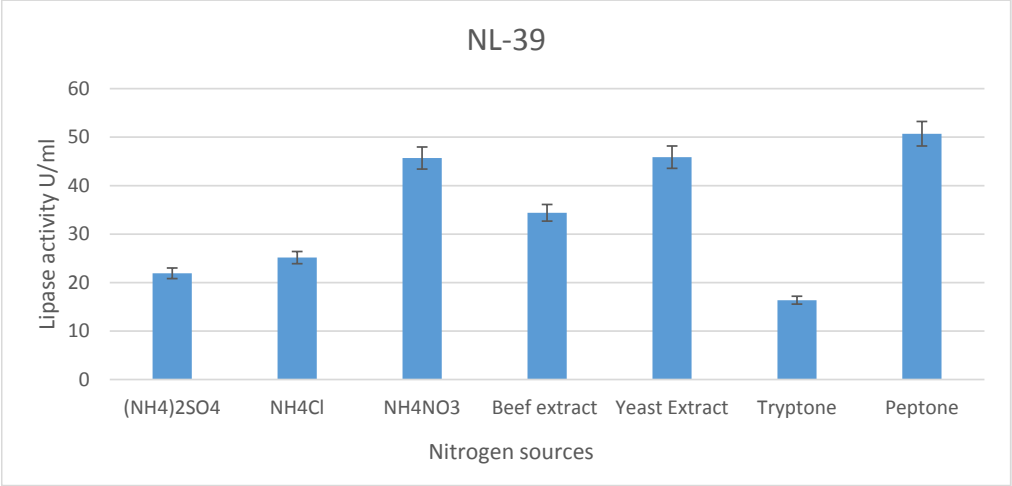

Figure 8. Effect of various nitrogen sources on lipase production.

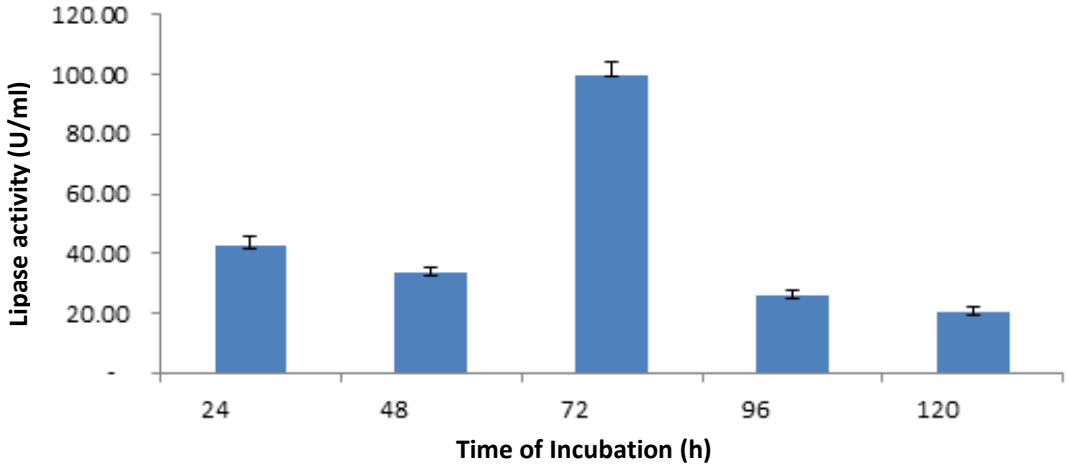

Figure 9. Effect of incubation time for lipase production.

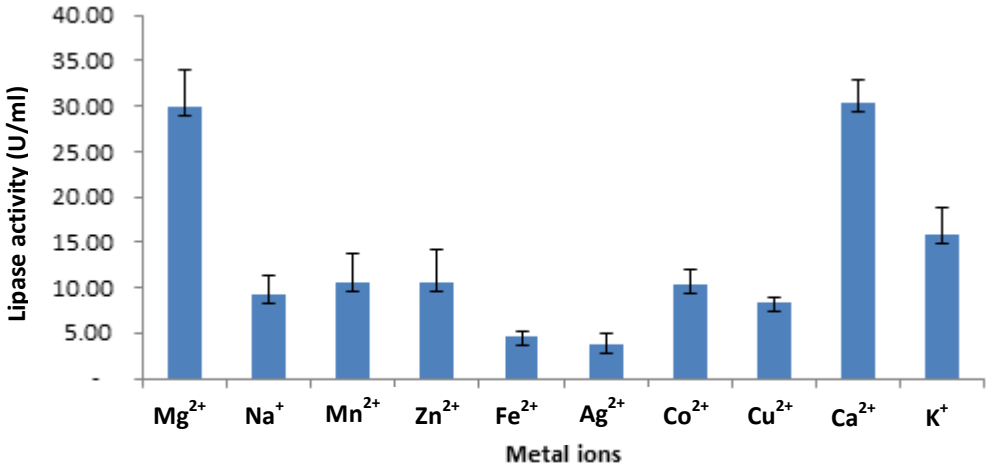

Figure 10. Optimization of metal ions for lipase production.

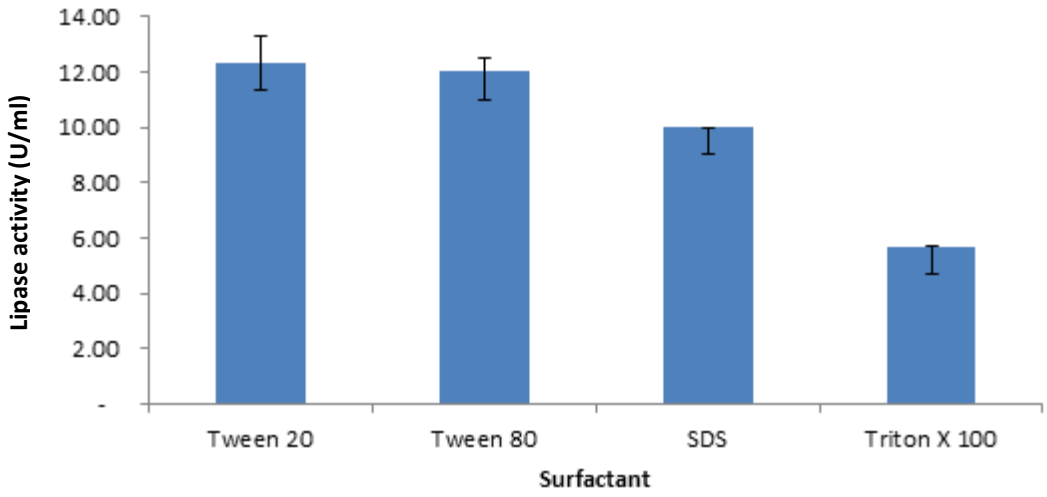

Figure 11. Optimization of detergent for lipase production.

Effect of detergents

Detergents like SDS, Tween 80, Tween 20 and triton 100 inhibit lipase production as illustrated in Figure 11.

Enzymatic activity

Lipase activity assay was carried out with help of Yamada et al. (1962) method. Lipase enzyme produced from PCSIR NL39 exhibited Vmax and Km of 101 µmol/min/mL and 7.6 mg, respectively (Figure 12).

DISCUSSION

Lipase constitutes a major group of biocatalysts, which have immense biotechnological applications. During the study, a lipase producing isolate PCSIRNL-39 was screened and identified as *B. subtilis* using 16S rDNA gene sequence analysis. 16S rDNA sequence obtained was aligned with Genbank sequences, using the BlastN program. This revealed a close relatedness to *B. subtilis* with 99% similarity, with an existing database. The identification of lipase producing *Bacillus* sp. by biochemical and 16S rDNA sequence analysis was done by different scientists (Akanbi et al., 2010; Kanimozhi and Perinbam, 2010; Prasad and Manjunath, 2012).

The bacterial ability to produce lipases is dependent on medium composition and various environmental factors such as temperature, pH, incubation time, etc. that greatly influenced the lipase synthesis and its activity, optimization of medium composition and other parameters for lipase production, which was performed in

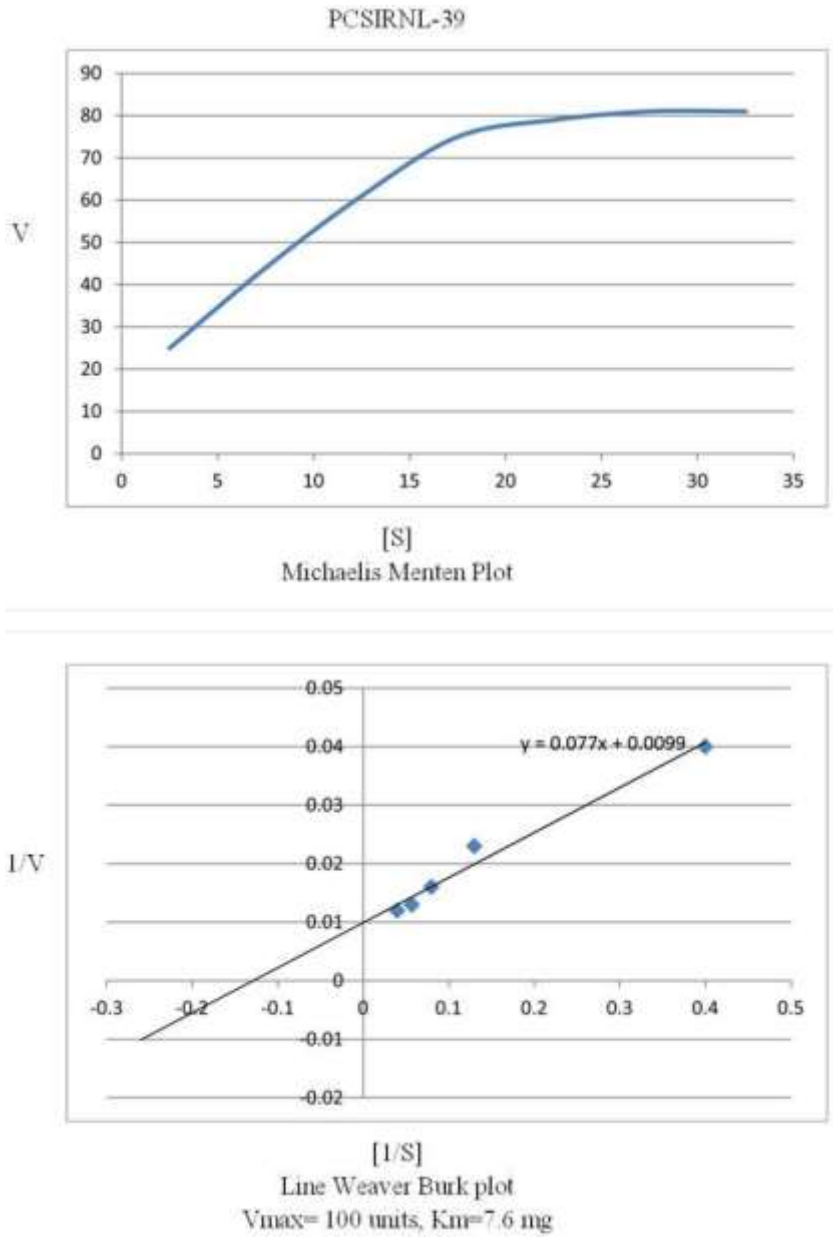

Figure 12. Vmax and Km calculations of lipase enzyme.

this study.

The study revealed that the best carbon sources for lipase production were fructose and sucrose. In addition to bagasse, during this study, whereas mannitol and glucose showed relatively low lipase production, the results are similar to Alabras et al. (2017). On the other hand, the type of nitrogen source in the medium also influences the lipase production (Ghosh et al., 1996). De-Almeida et al. (2016) has earlier shown lipase production by various substrates individually, and after mixing with oils like olive oil. In the present experiment, the best nitrogen sources were peptone and yeast extract, similar to the results shown by Hasan et al. (2001). Basically, organic nitrogen such as peptone and yeast extract, have preferably been used as nitrogen source for lipase production by various *Bacillus* spp. (viz. *Bacillus* strain A30-1, *B. alcalophilus*, *B. Licheniformis* strain H1), different pseudomonas (viz. *Pseudomonas* sp., *P. fragi*, *P. fluorescens* BW 96CC) and *Staphylococcus haemolyticeus* (Oh et al., 1999; Ghanem et al., 2000; Lanser et al., 2002; Sharma et al., 2002), while tryptone and yeast extract have been used for *S. haemolyticus* L62 (Oh et al., 1999).

In case of the present research, NH_4NO_3 as inorganic nitrogen source also showed maximum activity of 45 U/ml. Ammonium chloride and ammonium sulphate had

22.33 and 25.17 U/ml enzymatic activity, respectively. Songs et al. (2001) reported that *Candida rugosa* produced optimum lipase when ammonium nitrate is used as nitrogen source. According to the work reported by Markossian et al. (2000), *B. thermoleovoraus* showed maximum activity when yeast extract is used as nitrogen source. If the yeast extract is replaced by ammonium sulphate, no activity will be observed (Pimentel et al., 1994). Vmax and Km of PCSIRNL-39 lipase were measured to be 101 µmol/min/mL and 7.61 mg, whereas Vasiee et al. (2016) reported Vmax and Km value of 0.367 µM/min/mL and 5.3 mM, respectively.

Incubation periods ranging from a particular hour to several hours, was found to be the best for maximum lipase production, by bacteria. In this study, all *Bacillus* sp. showed maximum lipase production at 72 h and similar results were examined by Sarkar et al. (1998). In contrast, 12 h was the optimum incubation period for *A. calcoaceticus* and *Bacillus* sp. (Mahler et al., 2000) and 16 h the optimum incubation period for *B. thermocatenulatus (*Dannert et al., 1997). However, the maximum lipase activity was shown to be 72 and 96 h in the case of *P. fragi* and *P. fluorescens,* respectively (Pabai et al., 1996; Dong et al., 1999).

Optimum temperature for lipase production was observed with respect to optimum temperature for growth. In the case of *B. subtilis* PCSIRNL-39 investigated in this study, the optimum temperature was 45°C which is in agreement with the result of Alabras et al. (2017).

At times, the production of lipase is inhibited by calcium. In our study, Ca^{2+} and Mg^{2+} enhanced lipase production in contrast with Patkar and Bjorkling (1994) who showed the inhibitory effects of Zn^{2+} and Mg^{2+}.

Conclusion

The above study describes the optimized culture conditions such as temperature, pH, incubation time, metal ions, carbon and nitrogen sources for lipase production from newly isolated *B. subtilis* strain PSCIRNL-39, especially in an inexpensive medium. Lipases, produced from our isolate can be used as biodetergent and for bioremediation of waste water.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Persimmon leaf and seed powders could enhance nutritional value and acceptance of green tea

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Acceptance of green tea, a health promoting functional food material, could be increased if its flavor is improved. Persimmon seeds and leaves are likely potential options for green tea improvement since they contain a large number of volatile flavor compounds. In the present study, amino acids, volatile flavor compounds, and antioxidant potentials of persimmon tea (PT) prepared with green tea were investigated. PT contained 8 essential amino acids, most of which are associated with learning ability and memory as well as stroke and neurodegenerative diseases prevention. Total free amino acid was high in persimmon seed tea (273.37-300.61 µg/mL) as compared to persimmon leaf tea (179.75-198.44 µg/mL). Total phenol content of persimmon leaf tea ranged from 333.07 to 348.87 GAE µg/g and that of seed tea from 324.63 to 356.73 GAE µg/g. Some of the volatile flavor compounds such as 2-undecanone; furfural; 5-methyl furfural; benzoic acid, methyl ester; benzaldehyde, 4-methyl; and phenol, 2,4-bis (1,1-dimethylethyl) were found in persimmon leaf and seed tea. The results of the present study suggest that persimmon leaf and seed could offer good options to enhance the preference of green tea along with its nutritional value.

Key words: Acceptance, green tea, nutritional value, persimmon leaf, persimmon seed.

INTRODUCTION

Persimmon fruit contains different nutrients and phyto-chemicals which significantly contribute to its taste, color, nutritive, and medicinal values (Celik and Ercisli, 2007; Del Bubba et al., 2009). Not only the fruits but also other parts of persimmon like leaves and seeds are found to contain different phytochemicals having nutritive and medicinal values. Persimmon seeds possess high antioxidant potential and also contain high level of unsaturated fatty acids (Jang et al., 2010). Persimmon leaf extract showed therapeutic potential to alleviate the severity of radiation-induced liver injury, hyperglycemia, hypoinsulinemia, and dyslipidemia in rats (Ashry et al.,

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2016). Results of Huang et al. (2016) indicated that persimmon leaves contained a potent protective effect on cognitive deficits induced by amyloid beta in rats.

Applications of leaves and seeds of persimmon in preparing different food and beverage items have been practiced. Lim and Lee (2016) prepared persimmon leaf powder (PLP) supplemented cookies and found to improve them with the functional properties of PLP, without compromising on consumer acceptance.

Hirayama et al. (2016) suggested that intake of persimmon leaf extract would be useful for lowering blood pressure in subjects with high normal blood pressure and stage I hypertension and have no safety concern for long-term intake.

Very little information exists about the volatile profile of seed and leaf of Korean persimmon cultivars. On the other hand, the possible use of seed and leaf of Korean persimmon cultivars in preparing tea has not been studied. Consumption of tea has been associated with reduced incidence of chronic diseases, like cancer (Butt and Sultan, 2009) and cardiovascular diseases (Stangl et al., 2007). Green tea may have even more benefits due to higher levels of bioactive compounds than in black tea (Wang et al., 2011). However, green tea is less preferred because of its flavor as compared to fermented black and semi-fermented 'Oolong teas' (Han et al., 2016). Fruit flavor is resulted from a combination of taste and aroma (Klee, 2010). The taste mainly depends on sugars and organic acids, whereas the aroma on a large number of volatile organic compounds (Xi et al., 2016). As persimmon contains a large number of volatile compounds the objective of this study was to investigate the potential use of persimmon leaf and seed as a supplement of green tea. The volatile compounds present in the seed and leaf tea of two Korean persimmon cultivars of astringent type were investigated. Amino acid composition as well as antioxidant potential of the tea samples was also described.

MATERIALS AND METHODS

Chemicals and materials

Folin-Ciocalteu phenol reagent and 2,2-diphenylpicrylhydrazyl radical (DPPH) were procured from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade. Leaves and seeds; grown at Sangju Persimmon Experiment Station, Gyeonsangbuk-do, Korea; were obtained from the ready-to-eat maturity stage (November, 2015) of persimmon (*Diospyris kaki* Thunb. cv. Sangjudungsi and Sanggamdungsi). Green tea was obtained from a local store in Daegu, Korea in November of 2015.

Preparation of persimmon tea samples

Intact fresh leaves were harvested from the standing persimmon trees, kept into airtight plastic bags, and transported to the laboratory within 6 h of harvest. The leaves were washed with tap water, surface dried at room temperature, and freeze dried to make leaf powder. Freeze dried leaves were ground into powder using a ceramic ball mill (SW-BM117, Samwoo Engineering, Seoul, Korea) and strained using a series of US standard testing sieves (60 mesh) stacked in the portable sieve shaker (M-11630, Tyler Co., Salibury, NC, USA) for 10 min. The powder was collected, packed in zipper plastic bags, and stored at 4°C until analysis.

Seeds obtained from the healthy ready-to-eat stage persimmon fruits were washed with tap water to remove any adhering pulp and kept for hot air oven drying (60°C for 72 h). Dried seeds were ground using a roller mill (K-150, Hanil Co, Seoul, Korea) and seed powder were prepared as leaf powder.

Although persimmon contains a large number of volatile compounds, a commercial green tea (CGT) was added to improve the flavor of persimmon leaf and seed tea. Persimmon seed tea (PST) was prepared by mixing equal volume of seed powder and CGT and persimmon leaf tea (PLT) by mixing equal volume of seed powder and CGT. Two grams of persimmon seed or leaf powder were mixed with 2 g of CGT and the mixture (4 g) was extracted with 100 mL of boiling distilled water for 2 min. The extracts were filtered through 0.2 µm syringe filter (Waters, Milford, MA, USA) for further analyses. Persimmon tea samples of were named as SJLT, Sangjudungsi persimmon leaf tea; SJST, Sangjudungsi persimmon seed tea; SGLT, Sanggamdungsi persimmon leaf tea; and SGST, Sanggamdungsi persimmon seed tea.

Determination of free amino acid content of persimmon tea

Free amino acid composition was analyzed following the procedure of Je et al. (2005) with some modifications. Persimmon seed or leaf tea samples (3 g) were homogenized at 12000 rpm twice for 2 min with 20 mL of ice-cold 6% (v/v) perchloric acid in an ice bath using a homogenizer (HMF-985, Hanil). The homogenized sample was then incubated for 30 min in ice and centrifuged at 2000×*g* for 15 min. The residue was reextracted as described previously. The supernatants were combined and filtered through a Whatman No. 41 filter paper. The pH of the filtrate was adjusted to 7 using a 33% (w/v) KOH solution and centrifuged at 2000×*g* for 10 min to remove precipitate of potassium perclorate. The pH of the supernatant was adjusted to 2.2 with a 10 M HCl solution and then diluted to 50 mL with distilled water. Two milliliters of the extract was transferred into a clean tube and 1 mL of lithium citrate buffer (pH 2.2) was added to it. Samples were then analyzed using an automatic amino acid analyzer (Biochrom 20, Biochrom Ltd, Cambridge, UK).

Solid phase microextraction (SPME) procedures

SPME is a powerful sample preparation tool prior to mass spectrometric analysis. SPME parameters were optimized for extraction and desorption for persimmon tea volatiles. Ten milliliters of persimmon tea was added to a 40 mL glass vial containing a small Teflon-coated stirring bar with a screw top and Teflon-lined septum. After the equilibration time of 20 min, volatiles from the persimmon tea headspace were extracted for 30 min at 40°C using a 100 mm 50/30 µm DVB/Carboxen/PDMS SPME fiber (Supelco, Bellefonte, PA, USA). Before each exposure, the fiber was cleaned in a 260°C injection port for 5 min.

GC-MS

Gas chromatography – mass spectrometry, GC-MS (Clarus 500 quadruple, Perkin/Elmer, Shelton, CT, USA) analyses were equipped with a software (Turbo Mass, Perkin/Elmer). Conditions for GC-MS were as follows: Helium was used as the carrier gas with a constant flow mode of 2 mL/min. The source was kept at 200°C, and the transfer line and injector were kept at 220°C. Compounds were separated on a 60 m, 0.25 mm i.d., 0.5 µm DB-

Wax column (J&W Scientific, Folsom, CA, USA). The mass spectrometer was operated in the total ion chromatogram at 70 eV. Data were collected from 40 to 300 m/z. Mass spectra matches were made by comparison of NIST 2002 standard spectra (NIST, Gaithersburg, MD, USA).

Identification of volatile compounds

Initial identifications were based upon the matches made from spectra in the NIST (National Institute of Standards and Technology) library, aroma descriptors, and linear retention index matches from literature or from standards. The final confirmation was based upon the combined matching of retention indices (LRI values), full scan mass spectra values, and aroma descriptions from standards with those observed in the sample.

Determination of total phenol content of persimmon tea

The total phenol contents of persimmon seed tea were estimated according to the Folin-Ciocalteau method (Singleton et al., 1999). Fifty microliters of persimmon tea extract sample was mixed to 250 µL of Folin-Ciocalteau. After 1 min, 750 µL of aqueous solution of Na_2CO_3 (20%) was added, and the volume was made up to 5 mL using distilled water. The mixture was kept for 2 h at room temperature under dark condition and the absorbance value was measured at 760 nm using a spectrophotometer (Multiskan GO, Thermo Fisher Scientific Oy, Vantaa, Finland). Gallic acid was used to plot the standard calibration curve. The total phenol contents were determined as gallic acid equivalents (µg GAE/mL of sample), and values are reported as mean values of triplicate analyses.

DPPH radical scavenging activity

Antioxidant activity was measured with DPPH radical scavenging method according to Cheung et al. (2003) with modification. Eight hundred microliters of 0.2 mM DPPH ethanol solution was mixed with 0.2 mL of persimmon tea samples and kept for 30 min at room temperature under dark condition. The absorbance value was measured at 520 nm using a spectrophotometer (Multiskan GO, Thermo Fisher Scientific Oy).

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using SAS 9.4. Differences between means at $p<0.05$ were identified using Tukey test. Average values are presented as mean±standard deviation (SD).

RESULTS AND DISCUSSION

Free amino acid composition of persimmon tea

At least 19 free amino acids were found in all 4 tea samples; SJLT, SJST, SGLT, and SGST (Table 1). Total free amino acids content was higher in the seed tea samples than in leaf tea samples. Out of 19 free amino acids detected in all the samples, 6 were essential ones. Sixteen amino acids were not detected in either sample whereas L-citrulline and L-α-amino-n-butylric acid were detected only in seed tea samples of both cultivars and proline was found only in SGLT. Total free amino acids were highest in SJST (300.61 µg/mL) followed by SGST (273.37), SJLT (198.44), and SGLT (179.75). L-Aspartic acid (73.29-122.32 µg/mL) and L-Sarcosine (83.19-139.68) were the most abundant free amino acids found in the tea samples.

Amino acids γ-amino-n-butyric acid (GABA) and glycine were found in all the tea samples. These two amino acids are associated with the learning and memory, stroke and neurodegenerative diseases; mediate signals between neurons that inhibit neutral amino acids and thus relieve anxiety, sedation, anticonvulsant, and muscle relaxation functions (Mody et al., 1994; Oh and Oh, 2004). GABA rich foods are considered as brain food because of its bioactive capabilities to enhance blood cholesterol and triglyceride blood pressure suppression, improved cerebral blood flow, antioxidant, diuretic, insomnia, depression and anxiety stabilizing effect on nerves, and pain (Dhakal et al., 2012). The amount of amino acids is one of the key factors in determining the nutritional qualities of food materials (Basarova and Janousek, 2000). The variation in the amino acid content in persimmon tea samples might be due to variation in cultivar since pomological properties of fruit are strongly affected cultivar variation (Mratinic et al., 2011). Moreover, Park et al. (2003) found changes in organic nutrients of persimmon leaves by differing in the time of abscission.

Volatile flavor compounds of persimmon leaf and seed tea samples

Volatile flavor compounds identified in leaf tea are shown in Table 2 and those in seed tea are in Table 3. Nine volatile flavor compounds were detected in SJLT whereas SGLT contained 13 compounds. Volatile compounds, 1-octen-3-yl acetate; 3-heptafluorobutyroxypentadecane 3-trifluoroacetoxytridecane; 2-pentadecanone, 6,10,14-trimethyl; 3-heptanol; 2-hexanol, 2,5-dimethyl; and octane, 4-methyl were not detected in SJLT whereas hexadecamethyl; nonanoic acid; and benzoic acid were not detected in SGLT. Relatively low number of volatile flavor compounds was detected in seed tea of ether cultivar as compared to leaf tea (Tables 2 and 3). There were seven volatile flavor compounds identified in SJST whereas nine compounds were detected in SGST. The only compound that was detected in SJST but not in SGST was 3-(4-tertiobutylphenyl)-propanal. Compounds 5-methyl furfural; 2-furancarboxylic acid, methyl ester; and 1,4,7,10,13,16-hexaoxacyclooctadecane were detected in SGST but not in SJST.

Wang et al. (2012) identified 50 volatile and aroma-impact compounds from persimmon fruits of Triumph cultivar. Thirty-eight volatile compounds were detected using steam distillation from leaves of 3 persimmon cultivars; Fuyugaki, Fujigaki, and Jitsuseisibugaki (Kameoka et al., 1989). The difference in the volatile

Table 1. Free amino acid composition (μg/mL) of persimmon leaf and seed tea samples.

Amino acid	Sample[1)]			
	SJLT	SGLT	SJST	SGST
O-Phospho-L-serine	ND[2)]	ND	ND	ND
Taurine	ND	ND	ND	ND
O-Phospho ethanol amine	ND	ND	ND	ND
Urea	ND	ND	ND	ND
L-Aspartic acid	73.29[3)]	81.34	122.32	114.84
L-Threonine	0.89	0.92	1.38	1.34
L-Serine	2.28	2.40	3.61	3.49
L-Glutamic acid	8.61	9.43	14.81	13.66
L-Sarcosine	83.19	87.89	139.68	122.38
L-α-Aminoadipic acid	ND	ND	ND	ND
Glycine	0.71	0.73	0.73	0.79
L-Alanine	1.61	1.65	2.41	2.25
L-Citrulline	ND	ND	1.16	1.35
L-α-Amino-n-butylric acid	ND	ND	0.28	0.43
L-Valine	0.92	1.02	1.17	1.12
L-Cystine	2.79	3.23	4.95	4.29
L-Methionine	ND	ND	ND	ND
Cystathionine	ND	ND	ND	ND
L-Isoleucine	0.27	0.23	0.31	0.28
L-Leucine	0.67	0.63	0.74	0.71
L-Tyrosine	0.41	0.45	0.46	0.42
L-Phenylalanine	0.81	0.74	0.95	1.02
β-Alanine	0.05	0.05	0.08	0.06
D, L-β-Aminoisobutyric acid	ND	ND	ND	ND
γ-Amino-n-butyric acid	1.32	1.35	2.27	2.04
Ethanolamine	ND	ND	ND	ND
Ammonia	0.94	1.02	1.28	1.12
Hydroxylysine	ND	ND	ND	ND
L-Ornithine	ND	ND	ND	ND
L-Lysine	0.38	0.49	0.87	0.68
1-Methyl-L-histidine	0.37	0.42	0.59	0.59
L-Histidine	ND	ND	ND	ND
3-Methly-L-histidine	0.23	0.36	0.54	0.48
L-Anserine	ND	ND	ND	ND
L-Carnosine	ND	ND	ND	ND
L-Arginine	ND	ND	ND	ND
Hydroxy proline	ND	ND	ND	ND
Proline	ND	4.08	ND	ND
Total free amino acid	179.75	198.44	300.61	273.37

[1)]SJLT, Sangjudungsi cultivar's leaf tea; SGLT, Sanggamdungsi cultivar's leaf tea; SJST, Sangjudungsi cultivar's seed tea; SGST, Sanggamdungsi cultivar's seed tea. [2)]Not detected. [3)]Quoted values are average of duplicate experiments.

compound in the present study from those of previous ones might be due to variation in cultivar since pomological properties of fruit are strongly affected cultivar variation (Mratinic et al., 2011).

Incredible efforts have been made to comprehend the variations in the volatile constituents of green tea (Hara et al., 1995). Investigation on profiling the volatile compounds of green tea enhances the understanding of tea flavors specifically perceived by consumers (Schuh and Schieberle, 2006; Baba and Kumazawa, 2014). Studies on compelling odorants of green tea are crucial to the characteristic aroma of the tea (Kumazawa and

Table 2. Volatile flavor compounds identified in Sangjudungsi leaf tea (SJLT) and Sanggamdungsi leaf tea (SGLT).

RT[1] (min)	Compounds	Peak area (%)	
		SJLT	SGLT
7.942	1-Octen-3-yl acetate	ND[2]	1.1
8.039	2-Undecanone	4.2	6.51
8.436	3-Heptafluorobutyroxypentadecane 3-Trifluoroacetoxytridecane	ND	0.64
9.397	2-Pentadecanone, 6,10,14-trimethyl-	ND	0.8
11.88	Nonanol, trimethyl-	2.33	5.13
12.42	3-Heptanol	ND	2.06
12.76	2-Hexanol, 2,5-dimethyl-	ND	1.56
13.71	Octane, 4-methyl-	ND	1.8
19.79	Furfural	25.1	12.64
25.56	5-Methyl furfural	4.56	2.08
27.79	Benzoic acid, methyl ester	5.91	4.97
29.06	Benzaldehyde, 4-methyl-	15.9	12.1
55.51	Nonanoic acid	4.3	ND
62.05	Phenol, 2,4-bis(1,1-dimethylethyl)-	19	11.36
66.31	Benzoic acid	6.86	ND

[1]Retention time. [2]Not detected.

Table 3. Volatile flavor compounds identified in Sangjudungsi seed tea (SJST) and Sanggamdungsi seed tea (SGST).

RT[1] (min)	Compounds	Peak area (%)	
		SJST	SGST
8.039	2-Undecanone	5.86	5.51
11.87	Acrylic acid, 2-acetamido-	3.09	2.01
17.83	3-(4-tertiobutylphenyl)-propanal	4.64	ND
19.80	Furfural	6.58	11.57
25.56	5-Methyl furfural	ND[2]	2.00
25.86	2-Furancarboxylic acid, methyl ester	ND	4.05
27.79	Benzoic acid, methyl ester	9.58	6.78
29.06	Benzaldehyde, 4-methyl-	20.09	17.71
62.05	Phenol, 2,4-bis(1,1-dimethylethyl)-	30.15	22.40
70.67	1,4,7,10,13,16-Hexaoxacyclooctadecane	ND	3.66

[1]Retention time. [2]Not detected.

Masuda, 1999; Baba and Kumazawa, 2014). In such circumstances, use of persimmon leaves or seeds could enhance the preference of green tea by enriching its flavor as it is less preferred because of its flavor as compared to fermented black and semi-fermented Oolong teas (Han et al., 2016). Although the effect of persimmon leaf on sensory properties of green tea is not reported, addition of chopped persimmon leaves gave significantly high scores for color, flavor, texture and overall preference to kimchi, a Korean traditional famous food (Park et al., 2010).

Antioxidant activity of persimmon seed tea

Antioxidant potential of persimmon tea was measured by α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging activity and content of phenols (Table 4). The DPPH radical-scavenging potential of SGST was significantly (p<0.05) high as compared to the other 3 tea samples. Total phenol content of SGLT (348.87 µg/g) and SJST (356.73 µg/g) were significantly (p<0.05) higher than those of SJLT (333.07 µg/g) and SGST (324.63 µg/g).

Since DPPH method evaluates the concentration of radical-scavenging materials actively by a chain-breaking mechanism, it is considered as one of the most effective methods to determine the antioxidant activity of plant extracts (Niki, 1987). The results of DPPH study in this study showed that the leaf and seed tea of persimmon are good free radical scavengers. Results of Jang et al. (2010) and Akter et al. (2010) also showed that

Table 4. DPPH radical scavenging activities and total phenol contents of persimmon leaf and seed tea samples.

Sample[1)]	DPPH (% Inhibition)	Total phenol content (GAE[3)] μg/g of sample)
SJLT	84.89±0.02[b2)]	333.07±3.12[b]
SGLT	84.51±0.87[b]	348.87±6.00[a]
SJST	84.26±0.31[b]	356.73±2.11[a]
SGST	87.22±0.71[a]	324.63±0.88[b]

[1)]SJLT, Sangjudungsi cultivar's leaf tea; SGLT, Sanggamdungsi cultivar's leaf tea; SJST, Sangjudungsi cultivar's seed tea; SGST, Sanggamdungsi cultivar's seed tea..[2)]Values are the mean±SD of triplicate experiments. Different superscripts in the same column followed by the values are significantly different at $p<0.05$.[3)]Gallic acid equivalents.

persimmon seeds possess high antioxidant potential. High antioxidant potential of persimmon leaves has also been reported in previous studies (Martínez-Las Heras et al., 2014; Lim and Lee, 2016). The results of the present study showed that persimmon tea samples contain good antioxidant potentials. Difference in antioxidant properties of persimmon tea samples might be due to the difference in the amount and form of tannin, the main phenolic compound of persimmon (Jang et al., 2011). Kim et al. (2015) mentioned that persimmons contain a specific flavonoid, cyanidin which was not found in green tea. Astragalin (kaempferol-3-O-glucoside), a flavonoid having anti-tumor, anti-inflammatory, and antioxidant activity have been found from leaves of persimmon and green tea seeds (Burmistrova et al., 2011; Kim and Kim, 2011). This report implies that addition of persimmon leaf and or seed powder to green tea further enhances its antioxidant potential. In addition, tannins from persimmon were found to show more anti-viral effects against a broad range of viruses than those derived from green tea (Ueda et al., 2013).

In conclusion, persimmon tea samples contained 8 out of 9 essential amino acids needed for human. In addition, amino acids like γ-aminobutyric acid (GABA) and glycine; which are associated with the learning and memory as well as stroke and neurodegenerative diseases were found in all the tea samples. A considerable number of volatile compounds are found in persimmon tea samples. Green tea, which is considered as health promoting functional food material, is less preferred because of its flavor as compared to fermented black and semi-fermented. Use of persimmon leaves and or seeds showed potentiality of enhancing the preference of green tea by enriching its flavor. From the antioxidant perspective, adding persimmon leaf and or seed to the green tea is to enhance its antioxidant potentiality. The results of the present study showed that persimmon leaf and seed tea offer a good choice to enhance the preference of green tea as well as enrich its antioxidant potentials.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Traditional production technology, consumption and quality attributes of toubani: A ready-to-eat legume food from West Africa

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Toubani is a traditional ready-to-eat legume food, consumed as staple food with stews. This study investigated the socio-cultural profile of stakeholders, the traditional processing techniques and sensory quality attributes. For this purpose, a survey was carried out in two municipalities using a questionnaire managed to stakeholders. The results showed that toubani production is exclusively undertaken by women while consumption is by all classes of people. Three types of Toubani were identified, varying in their raw materials and processing technologies: the use of cowpea as a single element or in combination with yam. The sensory quality attributes of Toubani with regards to consumers' preference was linked to the perception of processors.

Key words: Toubani, cowpea, yam, quality attributes, production, consumption.

INTRODUCTION

Cowpea is the most economically important indigenous African legume crop. Cowpeas are of vital importance to livelihood of several million people in West and central Africa (Gómez, 2004). Cowpea is a plant source with high protein, containing 20 to 30 g of protein per 100 g of dry weight and very popular in West Africa. It also contains high concentrations of carbohydrate, vitamins and minerals (Abioye et al., 2015). Cowpea, legume grain (*Vigna unguiculata* (L) Walp), is the main source of income, it stands as the second most important starchy food for people after maize (Madodé, 2012). In Benin, this legume is an important component in the religious rites and is one of the sacred meal offering to the ancestral spirits (Dansi et al., 2008). Different traditional African meals and seasonings are made up of cowpea, among them are homemade weaning foods (Lambot, 2002). It is an important food for children as well as adults and is prepared in a variety form of dishes.

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Cowpea grains are often cooked alone (Abobo, Adowè) or ground into flour and made into cake (Ata, Akara) or steamed (Madodé et al., 2011; Taiwo, 1998). The consumption of this legume is particularly important in Benin since protein malnutrition is major health problem in West Africa. Grains legumes are low-cost source of protein than animal protein (Lateef et al., 2010; Egounlety and Aworh, 2003). In the North Benin, steam-cooked mixed cowpea with yam paste product is popularly called Toubani. This flavoury food is often consumed with spices and chopped onions or soup. There is however dearth of information on the processing technology, raw materials used and can lead to variation in nutritional composition of this food. To overcome these lacks, this investigation aims to gather information on the traditional processing techniques, problems associated with processing, consumption, and quality attributes of toubani through stakeholder knowledge and perception.

MATERIALS AND METHODS

Sampling of stakeholders and data collection

The survey was conducted in two localities in the North Benin, especially Parakou municipality (latitude 9°21'N and longitude 2°36'E) in the Borgou Department and Malanville municipality (latitude 11°12'N and longitude 2°28'E) in Alibori Department where toubani is commonly consumed. The sample size was determined according to Dagnelie (1998) as described by Chadare et al. (2008). Eligible interviewed stakeholders were selected by ranking toubani processors and consumers. A total of 140 people (40 processors-sellers, 100 consumers) from different socio-cultural groups and localities were interviewed.

Survey tool design

A questionnaire was designed to collect data on toubani production and consumption. Demographic data related to gender, age, marital status, and academic qualifications of processors were collected. Then, technical data on the process were gathered, including raw materials and ingredients used, type of toubani and constraints related to processing. Other information collected included sensory quality attributes of toubani, shelf life and safety problems. The survey was carried out through individual interviews or focus group discussions (2-4 interviewed) from both processors and consumers.

Statistical analysis

Survey data were entered in the Sphinx plus^2 software (Sphinx Survey Plus^2, Eureka) and were analysed for descriptive statistical patterns and trends. In addition, multivariate analyses were performed on the types of toubani and quality attributes as perceived by stakeholders using Statistica (version 7.1, StatSoft France, 2006).

RESULTS AND DISCUSSION

Socio-cultural profile of Toubani processors

The production and commercialization of toubani are exclusively female activities (100%) (Table 1). This is similar to what is generally obtained in Africa countries where women play very significant roles in traditional food production especially based on street food vendor activities. Similarly, Kindossi et al. (2012) also found women predominance amongst traditional processing technique for food. The processors surveyed were aged between 18 and over 50 years; 64.3% of them were between 18 and 30 years, while 35% are between 31 and over 50 years. These percentages show that the younger generation is more interested in this activity. From the survey, it was observed that the transmission of Toubani production technology is matrilineal; 90% of processors inherited the process techniques, the know-how and the remaining received the knowledge from their friend (10% of processors interviewed). The major socio cultural groups were Bariba (70%) followed by Nagot (18.5%), Dendi (8.5%) and Yom (3%). This confirms the general perception of consumers as to the origin of the product commonly recognized as a dish which draws out its source from bariba people who lived in the northern region of Benin. A relative proportion of processor-sellers of toubani (70%) were found to be illiterate, while the rest (30%) did not finish primary school. The production and commercialisation of toubani are an income generating activity for them. In Parakou and Malanville, toubani is sold in the morning and the evening at the edge of streets and other public places.

Table 1. Demographic characteristic of toubani processor and sellers.

Variables	Frequency (n=140)	Percentage
Ages (years)		
18-25	40	28.6
26-30	50	35.7
31-40	37	26
41-50	10	7
≥ 50	3	2
Gender		
Females	140	100
Educational status		
Illiterate (non-academic qualification)	98	70
Primary school	42	30
Socio cultural group		
Bariba	98	70
Nagot	26	18.5
Dendi	12	8.5
Yom	4	3

n: Number of processors interviewed.

Processing technique for Toubani

Raw materials and ingredients used

Raw materials used in toubani production include cowpea *(Vigna unguiculata*) singly or in combination with yam (*Dioscorea* sp.). The ingredients added to produce toubani were salt, potash, seasonings in various ratio according to processors. According to Madodé et al. (2011), certain types of potash modified the colour and can improve the iron content of the finished product, which was appreciated according to the region. Uzogara et al. (1988) clarified the change in colour obtained with the potash by a browning process due to the Maillard reaction and oxidation of the cowpea pigments. The preference for the use of these raw materials was 100% for cowpea processing and a mixture of cowpea with dried yam (55%). Similar information was gathered on moinmoin, a Nigerian steamed cowpea paste for which the cowpea grains were used singly or combined with maize (*Zea mays*) or with cassava flour (*Manihot esculenta*) (Olayiwola et al., 2012; Akusu and Kiin-Kabari, 2012; Ayode et al., 2012; Olapade and Adetuyi, 2007; Hongbété et al., 2011). In this study, the possible raw material combinations were dried yams. The mixing ratio of cowpea grains and dried yam differs from one locality to another and between processors within the same locality. This dissimilarity of ratio of cowpea and dried yam between localities and processors should affect the finished product quality. According to processors, the reasons for mixing the cowpea/yam included interest in improving consistency, increasing nutrient content and net profit improvement. Most processors (75%) used dried yam tuber (*Dioscorea rotundata*) of the kokoro type cultivar or dried yam tuber (*Dioscorea alata*) of Florido cultivar (25%). They reported that the yams from the kokoro group are more preferred by consumer than that of Florido. Also, other studies pointed out that yams tubers of kokoro group were mostly used for dried yam processing and its thick paste (amala) is generally the best preferred by consumers (Akissoé et al., 2001; Mestres et al., 2004). However, illegal substances such as fumigant type topstoxin and endosulfan (claimed by 33.3 and 29.3% of processors, respectively) are incorporated directly in cowpea grains and dried yams to control insects that attack during storage, while to ensure adequate dispersion, fumigants are often applied with air-moving equipment. Moreover, this practice observed during the survey can involve toxicity effect and extent contamination to stored products, finished products and even towards human beings.

Toubani is produced throughout the year with quantity of cowpea grains varying from 1 to 50 kg per week and per processor. Toubani is produced by majority of the respondents more than once a week. The processors (12.4%) of Toubani from cowpea and 25.9% from cowpea/yam produce two to three times per week, while 60% of processors of Toubani from cowpea grain, 68.9% from mixture cowpea/yam produce toubani more than three times per week.

Technology of Toubani production

Traditional toubani processing operations were reported to be laborious and time consuming. Three processing techniques were identified, these are toubani from non dehulled cowpea (ndc), toubani from dehulled cowpea (dc) and toubani from mixture cowpea and yam (c/y). Cowpea is processed into toubani using the diagram shown in Figure 1. The cowpea grains are sorted, cleaned of dust, sand and plant debris. The cleaned grains are washed and steeped in water at room temperature (26 to 35°C) to soften the hull to ease its removal in the production of dehulled flour (Figure 1A). This is however skipped from production of non dehulled cowpea flour (Figure 1B) and mixed cowpea/yam flour (Figure 1C). The dehulled steeped grains are drained and sun dried for 4 days. The dried dehulled cowpea grains, non dehulled cowpea grains or mixture cowpea grains with dried yams are milled followed by kneading (whipping) of each flour with water. During the kneading, potash, salt are added and homogenised. The resulting dough is then packaged using different recycled materials like milk tin, tomato tin, plastic cup. The packaged dough is then steam-cooked for 15 to 30 min.

Constraints related to processing

The constraints to toubani production were identified as sorting and cleaning, the laboriousness of the dehulling step and the kneading. Millers frequently refuse grinding because of the residual and undesirable beany odour on other miller products later. Mill need to be cleaned thoroughly after cowpea grinding, and so cleaning costs were indirectly demanded by extra charge.

Shelf life of toubani and safety problems

The shelf life of toubani varies depending on the process and the quality of the raw material and ingredients. Toubani processors (94.3% respondents) reported that toubani is produced daily for immediate consumption. So, it is complicated for them to preserve it for more than 24 h at ambiant temperature. The toubani is cooked by steaming, it could be free from any biologically damaging agent if it is well packaged with adequate packaging, its shelf life should not be short as observed from the survey. Similar short shelf life was recorded among African traditional dishes such as kunu and cowpea dishes (Gaffa et al., 2002; Madodé et al., 2011).

Additionally, the safety problems recorded with toubani

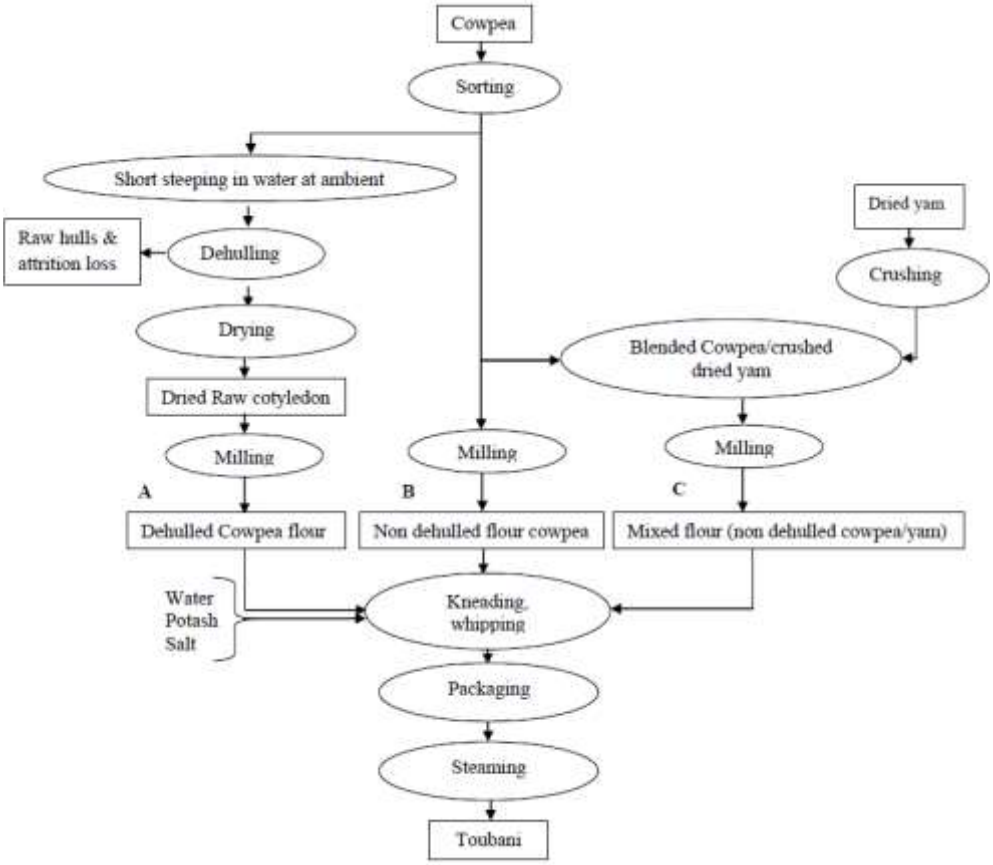

Figure 1. Flow sheet of toubani processing.

traditional production are related to the variability of theunit operations and the unhygienic condition of the production environment. The steeping and dehulling operations were not practised by certain processors while these operations are going on. Though the dehulling is a tedious and time consuming operation, it involves the removal of the hulls of cowpea grains steeped by presenting significant points such as reduction of tannin content, good appearance, high texture, cooking quality and digestibility of the grains (Clark et al., 2014). Elsewhere, the steeping, dehulling and drying parameters (temperature, duration and moisture) vary within and between processors. Also, the dehulled cowpea grain is sun dried, so the accomplishment depends on the weather, generally on the sunshine intensity. Furthermore, the cooking time and the quantity of ingredients varied from one processor to another. Therefore, there is a need for the process standardization.

Forms and frequency of toubani consumption

For consumers (62.8% of female and 37.2% of male, aged between 18 and 60), toubani is a ready-to-eat staple food eaten with stew or blend of oil, pepper and pieces of fresh onion. Most consumers frequently eat non dehulled cowpea toubani (19.6-28.6 %), dehulled cowpea toubani (1.3-32.7 %) and mixture of cowpea/yam toubani (5.3 to 38.3%) once or several times per week (Table 3). With regards to the occasion of consumption, these products are consumed at breakfast (25.3 to 28.5%), lunch (25.1 to 47.6%), and dinner (15.0 to 20.8%).

Quality perception of toubani

Processors and consumers gave their opinion on the significant quality attributes based on their observations for toubani, these are appearance, taste, colour, soft to the feel, smooth texture, springiness and beany flavour, etc. (Table 2). According to the two stakeholders (> 50% of respondents), toubani is recognized as being juiciness, salty in taste, have springiness, beany taste and beany flavour. Then, toubani made from cowpea alone is supposed to be slightly hard for 50.1 and 46.3% of the processors and 56.3 and 52.6% of consumers for non dehulled and dehulled cowpea toubani, respectively. Non-dehulled cowpea toubani and mixed cowpea/yam toubani should have a brownish colour (91.4 and 76.7%

Table 2. Sensory quality attributes of each type of toubani according to stakeholders (%).

Sensory attributes of Toubani	Processors			Consumers		
	non dehulled Cowpea (n= 20)	Dehulled cowpea (n=10)	mixture cowpea/yam (n=10)	Non dehulled Cowpea (n=80)	Dehulled cowpea (n=15)	mixture cowpea/yam (n=35)
Ashen colour	48.6	74.3	10.0	49.8	72.6	19.7
Brownish colour	91.4	37.1	76.7	85.3	23.3	82.3
Beany taste	78.6	82.9	50.0	77.9	82.5	42.2
Juiciness	82.6	65.8	74.4	94.5	74.1	84.4
Salty taste	75.3	70.0	71.2	85.3	80.1	82.3
Soft to the feel	52.7	42.7	91.7	61.5	39.6	96.6
Smooth texture	35.9	81.4	46.7	28.5	62.5	67.9
Springiness	50.9	58.5	89.6	54.5	61.4	86.7
Slightly hard	50.1	46.3	18.5	56.3	52.6	31.6
Mouth feel	13.6	45.9	71.7	18.2	51.1	81
Beany flavour	82.9	64.3	46.8	93.1	72.6	51.1
Very well cooked	99.0	98.0	100	100	100	100

n: Number of stakeholders interviewed.

Table 3. Consumption frequency of toubani (%).

Toubani	Non-dehulled cowpea	dehulled Cowpea	Mix cowpea /yam
Consumption frequency (times per week)			
Six-seven	19.6	1.3	5.3
Four – Five	24.1	27.4	17.2
Two –three	28.2	30.2	38.3
Once	24.6	32.7	24.4
Rarely	3.5	5.4	13.1
Occasion of consumption			
Breakfast	25.3	27.4	28.5
Lunch	47.6	25.1	39.4
Dinner	17.7	15.0	20.8

of processors and 85.3 and 82.3% of consumers, respectively). The Toubani of dehulled cowpea is considered to have an ashen colour (74.3% for processors and 85.3% for consumers) and smooth texture (81.4% producers and 62.5% of consumers). Also, mixed cowpea/yam toubani is softer to the feel (91.7% of processors and 96.6% of consumers) than other toubani from cowpea alone.

The relationship between the sensory quality attributes and stakeholders is illustrated by the principal component analysis (PCA) plot in Figure 2. The PCA revealed that 85.06% of variability in quality attribute perception could be explained by the first two axes, as 52.41% by the first principal component (Axis 1) and 32.65% by the second one (Axis 2) (Figure 2). All processors and consumers agreed on the required qualities attributes of Toubani. The PCA plot indicated that consumers were directly aligned to the perception of processors on quality criteria of each type of Toubani. So, the PCA shows three groups of stakeholders in relation to perception of sensory quality attributes of Toubani (Figure 2): the first relates to processors and consumers of Toubani (Pc/y and Cc/y) from mixed cowpea/yam (34.17%); the second group contains processors and consumers of toubani (Pdc and Cdc) from dehulled cowpea (29.65%) and the third group, processors and consumers of toubani (Pndc and Cndc) from non dehulled cowpea (24.30%). The PCA indicated that the preference of consumers was linked to the perception of processors. Each group seem to have a specific perception of the quality criteria of toubani. Then, for processors and consumers, dehulled cowpea toubani should have ashen colour, whereas non dehulled cowpea toubani should be slightly hard and the mixed cowpea/yam toubani should be soft to the feeling, have

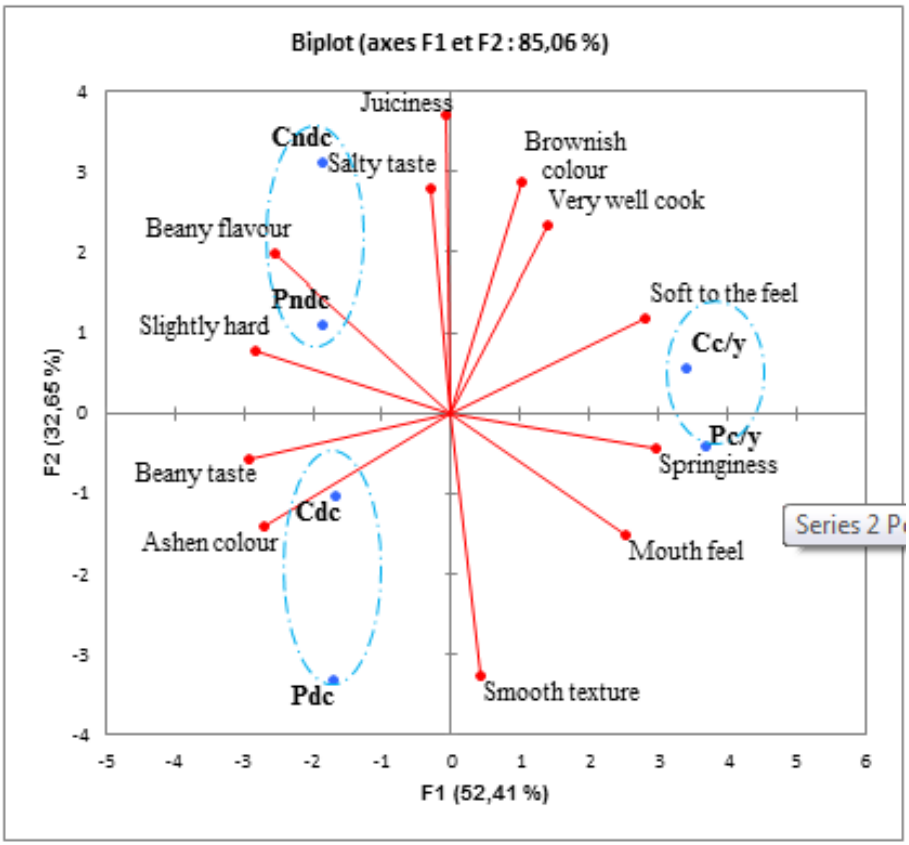

Figure 2. Relation between sensory quality attributes of toubani, linking to stakeholders characteristics. Pndc: Processors of toubani from non dehulled cowpea; Pdc: processors of toubani from dehulled cowpea; Pc/y: processors of toubani from blended non dehulled cowpea and yam Cndc: consumers of toubani from non dehulled cowpea; Cdc: consumers of toubani from dehulled cowpea; Cc/y: consumers of toubani from blended non dehulled cowpea and yam.

springiness and mouth feel. In addition, popular processors of toubani have a significant experience and particular knowledge on production by using more salt and prepared for with long cooking time. Also, the PCA revealed that some quality attributes were linked (Table 4). As shown in the result, the slight hardness of dehulled cowpea toubani was significantly and positively correlated with ashen colour ($r = 0.83$, $p < 0.05$), with beany taste ($r = 0.92$, $p < 0.05$) and beany flavour ($r = 0.90$, $p < 0.05$); and significantly and negatively correlated with soft to the feeling ($r = -0.82$, $p < 0.05$), with springiness ($r = -0.93$, $p < 0.05$) and mouth feel ($r = -0.79$, $p < 0.05$). These correlations indicate that the absence of cowpea hull impact ashen colour on the finished product but inhibited its softening. The salty taste was significantly and positively correlated with juiciness ($r = 0.82$, $p < 0.05$) and beany flavour ($r = 0.46$, $p < 0.05$). This correlation indicates that the salt and potash were used to improve water uptake, tenderness, succulence, cooking time and digestibility of the toubani. In addition, salt is retained as a key interaction with bean flavor that acts as positive driver. It was reported that salt reduces the water activity, and decreases the microbial proliferation. Also, salty taste enhances the bean flavor perception like that of meat, fish, which is an important factor in overall acceptability (Kindossi et al., 2013; Pietrasik and Gaudette, 2014). These correlations and the large range of sensory quality attributes, as well as the consumers' preferences will be helpful in upgrading the technology and improvement of the quality of toubani.

Conclusion

This investigation contributes to understanding on indigenous knowledge on toubani production and consumption. It shows that toubani is a ready to eat staple food produced from steam cooked cowpea used alone or in combination with yam. Toubani is commonly processed by the following unit operations: milling, kneading or whipping and cooking. Moreover, a large sensory quality attributes perceived by processors and

Table 4. Correlation coefficients between quality attributes of toubani.

Variables	Ashen colour	Brownish colour	Beany taste	Juiciness	Salty taste	Soft to the feel	Smooth texture	Springiness	Slightly hard	Mouth feel	Beany flavour	Very cook
Ashen colour	**1**											
Brownish colour	-0.696	**1**										
Beany taste	**0.920**	-0.468	**1**									
Juiciness	-0.310	0.715	-0.149	**1**								
Salty taste	-0.023	0.240	-0.053	**0.821**	**1**							
Soft to the feel	**-0.966**	0.615	**-0.974**	0.301	0.110	**1**						
Smooth texture	0.281	-0.680	-0.076	-0.745	-0.367	-0.109	**1**					
Springiness	-0.811	0.166	**-0.933**	-0.130	-0.117	**0.870**	0.257	**1**				
Slightly hard	**0.828**	-0.249	**0.863**	0.265	0.417	**-0.822**	-0.187	**-0.931**	**1**			
Mouth feel	-0.525	-0.187	-0.779	-0.367	-0.148	0.646	0.592	**0.919**	-0.794	**1**		
Beany flavour	0.570	0.106	0.759	0.526	0.459	-0.635	-0.577	**-0.897**	**0.904**	**-0.934**	**1**	
Very cook	-0.538	0.272	-0.495	0.566	0.649	0.529	-0.440	0.467	-0.246	0.309	-0.081	**1**

Coefficient in bold are significant to P<0.05.

consumers should be useful for standardizing the processing technique of toubani.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Juice clarification with tannases from *Aspergillus carneus* URM5577 produced by solid-state fermentation using *Terminalia catappa* L. leaves

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Tannase is an inducible extracellular enzyme produced by filamentous fungi, yeasts, and bacteria by solid-state fermentation (SSF) or submerged fermentation (SmF). Among the filamentous fungi, *Aspergillus* and *Penicillium* are recognized as the most efficient producers of tannase. The aims of this study were to evaluate the production of tannase under SSF by isolation from *Aspergillus* and *Penicillium* preserved in the Collection of Cultures Micoteca - URM (WDCM604); select the best enzyme producer, and use the crude extract to clarify mangaba and tamarind juices. The optimal conditions were determined by using the Placket-Burman Planning (PB) and response surface methodology (RSM). All tested crops produced activity between 2088.19 and 238.93 U/gds, and *Aspergillus carneus* URM5577 was the best producer. Through MSR, the best parameters for producing tannase were found to be 70 h of cultivation at pH 6.0, 7% tannic acid at 28°C and, as the response variable, 5449.31 activity U/gds. The optimum purification conditions were the molecular weight of PEG 8000 (g/mol), concentration of PEG 15% (w/w), 25% citrate (w/w), and pH 8.0. Its application in mangaba juice reduced the tannin content by 49.66% after 90 min and in tamarind by 51.82% at 120 min incubation at 37°C.

Key words: Tannase, agroforestry waste, *Aspergillus, Penicillium.*

INTRODUCTION

Fungi are organisms responsible for manufacturing various highly important products, such as food, beverages, organic acids, drugs etc. Among the fungi commonly preserved in culture collections are those belonging to the genera *Aspergillus* and *Penicillium* (Bon et al., 2008).

Tannins occur in a wide variety of vegetables and can be found in every part of the plant. They represent the fourth most abundant constituent of plants after cellulose, hemicellulose, and lignin - compounds that are degraded by the action of tannase (Yadav et al., 2008). Tannin acyl hydrolase (TAH) (EC 3.1.1.20), or tannase, is an inducible

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enzyme that catalyzes the hydrolysis of ester bonds and hydrolysable tannins in peptide such as tannic acid, releasing glucose and gallic acid (Pinto et al., 2005; Costa et al., 2008). The filamentous fungi are recognized as great producers of this enzyme, and species of the genera *Aspergillus* and *Penicillium* stand out in this respect (Costa et al., 2008; Cruz et al., 2013; Lima et al., 2014).

The filamentous fungi are featured in fermentation processes, as they can secrete substantial quantities of proteins in culture media (Gomes et al., 2007). There are two types of fermentation, submerged fermentation (FmS), in which free water is present and solid-state fermentation (SSF), wherein the microorganism is usually inoculated waste or solid substrates in the absence of free water.

Several types of substrates are used in the production of fungal tannase, which aim at reducing production costs. The "castanhola" leaves (*Terminalia catappa* L.) is rich in tannins but has never been used as a substrate to produce tannase. When used in the production of these enzymes, these substrates can achieve a high market value (Selwal and Selwal, 2012; Cruz et al., 2013).

The presence of tannin gives food a bitter taste, decreasing its consumption and consequently, its commercial value. The use of tannase to clarify tannin-containing drinks may be a solution to these problems (Lima et al., 2014; Banerjee et al., 2005).

Depending on the final application, methods of tannase purification may be sought as alternatives to extraction with organic solvents, such as a pre-purification aqueous two-phase system (ATPS). The aqueous two-phase system is formed by the meeting of certain polymers, polyelectrolytes, or polymers in combination with low-molecular-weight solutes. This process can be used to purify tannase for use in the clarification of juices or in the production of animal feed (Luccarini et al., 2005).

Therefore, the purpose of this work was to evaluate the production of tannase under SSF by isolation from *Aspergillus* and *Penicillium* preserved in the Culture Collection Micoteca - URM using "castanhola" leaves (*T. catappa* L.) as a substrate and to select the best producer, optimize the production, and apply the enzyme to the clarification of mangaba (*Hancornia speciosa* Gomes) and tamarind (*Tamarindus indica* L.) juices.

MATERIALS AND METHODS

Substrate

"Castanhola" leaves (*T. catappa* L.) were obtained on the campus of the Federal University of Pernambuco, located in the city of Recife in Pernambuco - Brazil. The leaves were previously washed with sterile distilled water and dried at 55°C for 72 h (Lima et al., 2014).

Microorganisms and inoculum preparation

In the present study, 30 species were used. Of these, 15 were species of *Aspergillus,* and 15 were of *Penicillium* preserved in mineral oil (Sherf, 1943) and maintained by the Collection of Cultures Micoteca - URM (WDCM604), from the Center of Biosciences of the Federal University of Pernambuco, Brazil.

Each strain was inoculated on malt extract agar (MEA) and incubated at 30°C. Subsequently, spores of each culture were transferred to a test tube containing 10 ml of sterile distilled water and 0.1% (v/v) Tween 80. The spore suspension was used as inoculum. The spores were quantified by the plate count technique, at a concentration of 5×10^8 spores/ml (Sabu et al., 2005).

Production of Tannase by solid-state fermentation (SSF)

Five grams of "Castanhola" leaves (*T. catappa* L.) were placed in 250-mL Erlenmeyer flasks and sterilized at 121°C for 30 min in flowing steam. The substrates were moistened with 5 mL of sterile salt solution containing 0.5% w/v NH_4NO_3, 0.1% w/v $MgSO_4.7H_2O$, and 0.1% w/v NaCl, pH 5.0. The moisture content was adjusted to 50%. Each vial was inoculated with 1 mL of spore solution (5×10^8 spores/mL). The contents were mixed and incubated at 30°C for 96 h. After this period, to each bottle was added 50 mL of distilled water containing 0.01% previously sterilized Tween 80. Then, the vials were shaken in a rotary shaker (Tecnal TE421, Sao Paulo, Brazil) at 150 rpm for 10 min. Then, the contents were filtered using Whatman number 1 filter paper, and the filtrate was regarded as crude enzyme extract, packaged in conical vials, and preserved at 4°C for later analysis (Sabu et al., 2005).

Determination of tannase activity

Tanase activity was measured by spectrophotometry (Sharma and Gupta, 2013). This method is based on the formation of a chromogen from gallic acid (released by the esterase activity of tannase) and rhodanine (2-thio-4- ketothiazolidine). To determine the gallic acid concentration, 100 µL crude enzymatic extract was incubated with tannic acid (0.3 mM) and sodium phosphate buffer (10 mM, pH 5.5) for 30 min at 30°C. Then, 300 µL of the methanolic solution of rhodanine (0.667% w/v rhodanine in 100% methanol) and 100 μL of 500 mM KOH were added to the mixture, which was diluted with 8.6 mL distilled water and incubated for 10 min at 30°C. After this period, samples were read in a spectrophotometer (Hitachi-U5100) at a wavelength of 520 nm. A standard curve was generated using gallic acid in different concentrations. All assays were performed in triplicate. One unit of tannase activity (U) was defined as the amount of enzyme required to release one micromole of gallic acid per minute under the defined reaction conditions. Enzyme yield was expressed in U/gds.

Statistical analysis

Optimization of the production of tannase

To optimize the conditions for the production of tannase from *A. carneus* URM5577 Plackett-Burman (PB) planning and response surface methodology (RSM) design were used.

Identification and selection of the most important variables in the optimization using Plackett-Burman design planning (PB)

For selection of medium components to produce tannase, a PB design was used, in which the variables time (h), temperature (°C), pH, and tannic acid (%) were evaluated. Each component was examined on two levels: "−1" down and level "+1" tall, using the statistical software package STATISTICS 8.0 (Statsoft, 2008), which generated a set of 12 experimental tests.

Table 1. Levels of factors used in the experimental design of the selected type 24 for the extraction and purification of tannase using the ATPS PEG/phosphate.

Variable	Levels		
	Low (-1)	Centric (0)	High (+1)
M_{PEG}[a]	3350	6000	8000
C_{PEG}[b]	15	20	25
C_{FOS}[c]	15	20	25
pH	6.0	7.0	8.0

[a]PEG molecular weight (g/mol); [B]concentration of PEG (%); [C]concentration of phosphate (%).

Optimization of the selected components using response surface methodology (RSM)

To determine the optimal level of the two variables selected by Plackett-Burman design planning [time (h) and tannic acid (%)], a central composite design (CCD) was applied using the statistical software STATISTICS 8.0 (Statsoft, 2008). The experiments were conducted in a 250-ml Erlenmeyer flask containing 5 g of "Castanhola" leaves (*T. catappa* L.) moistened with saline solution (pH 6.0) prepared in accordance with the design, at 28°C.

Pre-purification of tannase

Preparation of an aqueous two-phase system

For tannase extraction, the system was prepared with polyethylene glycol (PEG) of different molecular weights (3350, 6000 and 8000 g/mol) and phosphate salts. The phosphate solution (40% w/w) was prepared by mixing appropriate amounts of dibasic potassium phosphate (K_2HPO_4) at different pH values (6.0, 7.0 and 8.0) at 25 ± 1°C. The desired amounts of PEG and salt were placed in graduated centrifuge tubes (15 ml). The crude enzyme extract containing PG represents 20% of the total system volume and was added to the tubes, and water was added to a final weight of 10 g. After 1 min of vortexing, the tubes remained at rest for 60 min to separate the phases. The volume of each phase was measured, and the phases were separated by using automated pipettes. The enzyme activities and dosage of the protein were determined, allowing the calculation of the partition coefficient, yield, and purification factor of tannase.

Experimental design

The effects of the molar mass of PEG (MPEG), the PEG concentration (CPEG), phosphate concentration (CFOS), and pH of the system response variables (partition coefficient (K), performance at work (Y), and purification factor (FP) were evaluated based on the results of a type-24 experimental design, another central point that was carried out twice to allow estimation of experimental error (Vieira Neto, 2002). The selected values for these variables (Table 1) were chosen based on the binodal diagrams in published reports (Vernau and Kula, 1990; Assis et al., 2001). All statistical analyses and graphs were generated by using Statistica 8.0 software (Statsoft, 2008).

Determination of partition coefficient, activity yield, purification factor, and selectivity

K was defined as the ratio of the volumetric enzyme activity in the top phase (At) to that in the bottom phase (Ab):

$$K = At/Ab$$

PF was calculated as the ratio of the specific activity in the top phase to the specific activity in the cell extract before partition:

$$PF = (At/Ct)/(Ai/Ci)$$

Where, Ct and Ci are the total protein concentrations, expressed in mg/mL, in the top phase and initial extract, respectively.

Y was defined as the ratio of total activity in the top phase over that in initial extract expressed as a percentage:

$$Y = [(At \times Vt)/(Ai \times Vi)] \times 100$$

Where, Vt and Vi are the volumes of the top phase and the initial extract, respectively.

Application of crude enzyme extract to mangaba juice and tamarind for clarification

For the preparation of juice, fruit mangaba (*H. speciosa* Gomes) and tamarind (*T. indica* L.) was washed in running water, the seeds were removed and pulp was liquefied (Black & Decker, LF910) and then filtered with the aid of a particle-size sieve (strainer in stainless steel–ASTM 1/4 inch opening 6.30 mm). The juice was stored at -4°C for further analysis (Sabu et al., 2005).

To clarify juice in 125-mL Erlenmeyer flasks, different aliquots of crude enzymatic extract: (0.5, 1.0, 1.5 and 2.0 mL), which contained 5449.31 U/gds tannase activity, were added separately to 10 mL of mangaba and tamarind juices. As a control, we used mangaba and tamarind juice separately, without the addition of crude enzyme extract. Then, the vials were placed on a rotary shaker at 150 rpm and 37°C for 120 min and analyzed every 30 min (0, 30, 60, 90 and 120 min). Assays were performed five times. After stirring, the flasks were incubated in a water bath for 10 min at 50°C. After this period, 1 mL juice treated with crude extract was removed to determine the concentration of enzyme extract tannins. The tannin content present in grapes was determined by the method of protein precipitation by tannins (Hagerman and Butler, 1978).

RESULTS

All 30 strains tested produced tannase through SSF (Table 2). The three best producers were *Aspergillus aureolus* URM 7034 (2088.19 U/gds), *A. carneus* URM 5577 (1128.59 U/gds) and *Penicillium implicatum* URM 6233 (1055.75 U/gds), respectively.

Two species have excelled in the production of the enzyme, *A. aureolus* URM 7034 and *A. carneus* URM 5577, these being selected to optimize the production of tannase.

Tannase production conditions for the two species mentioned above were optimized using the PB experimental design and after selecting the optimal conditions, RSM was applied, to select important variables in the production of tannase and to verify their significant levels. Four variables were analyzed: time (h), temperature (°C), pH, and tannic acid (%) (Table 3) in relation to the production of tannase. The effect of each

Table 2. Tannase activity (U/gds) of strains of *Aspergillus* and *Penicillium* in SSF, using "castanhola" leaves (*Terminalia catappa* L.) as substrate, after 96 h of fermentation.

Species	N° URM	U/gds
Aspergillus aureolus	7034	2088.19
A. carneus	5577	1128.59
Penicillium implicatum	6223	1055.75
P. brasilianum	6892	974.16
P. melinii	6463	933.37
A. viride-nutans	7033	929.49
P. corylophilum	6491	925.60
P. brevicompactum	6833	881.90
P. aurantiogriseum	6844	867.33
P. fellutanum	6472	855.67
A. candidus	6607	810.02
A. flavus	7028	802.25
A. flavo-furcatis	6142	774.09
P. griseofulvum	6846	773.12
A. versicolor	7029	749.81
A. carbonarius	6613	738.15
A. tubingensis	6991	683.76
P. adametzii	7015	662.39
P. janczewskii	6672	599.26
P. citrinum	7030	588.58
A. terreus	3420	523.50
A. aculeatus	7013	518.65
A. caespitosus	5938	511.85
A. sclerotiorum	6619	506.02
P. commune	6671	427.35
P. purpurogenum	6634	372.96
A. oryzae	5638	303.03
P. citreonigrum	6458	255.44
P. restrictum	6135	253.50
A. niveus	5461	238.93

Table 3. Experimental Plackett-Burman Matrix planning (CP) for producing tannase from *Aspergillus aureolus* URM 7034 and *Aspergillus carneus* URM 5577 through SSF using "castanhola" leaves.

Test	pH	Incubation temperature (ºC)	Tannic acid (%, p/v)	Fermentation time (h)
1	6 (+)	28 (-)	5 (+)	48 (-)
2	6 (+)	32 (+)	1 (-)	96 (+)
3	4 (-)	32 (+)	5 (+)	48 (-)
4	6 (+)	28 (-)	5 (+)	96 (+)
5	6 (+)	32 (+)	1 (-)	96 (+)
6	6 (+)	32 (+)	5 (+)	48 (-)
7	4 (-)	32 (+)	5 (+)	96 (+)
8	4 (-)	28 (-)	5 (+)	96 (+)
9	4 (-)	28 (-)	1 (-)	96 (+)
10	6 (+)	28 (-)	1 (-)	48 (-)
11	4 (-)	32 (+)	1 (-)	48 (-)
12	4 (-)	28 (-)	1 (-)	48 (-)

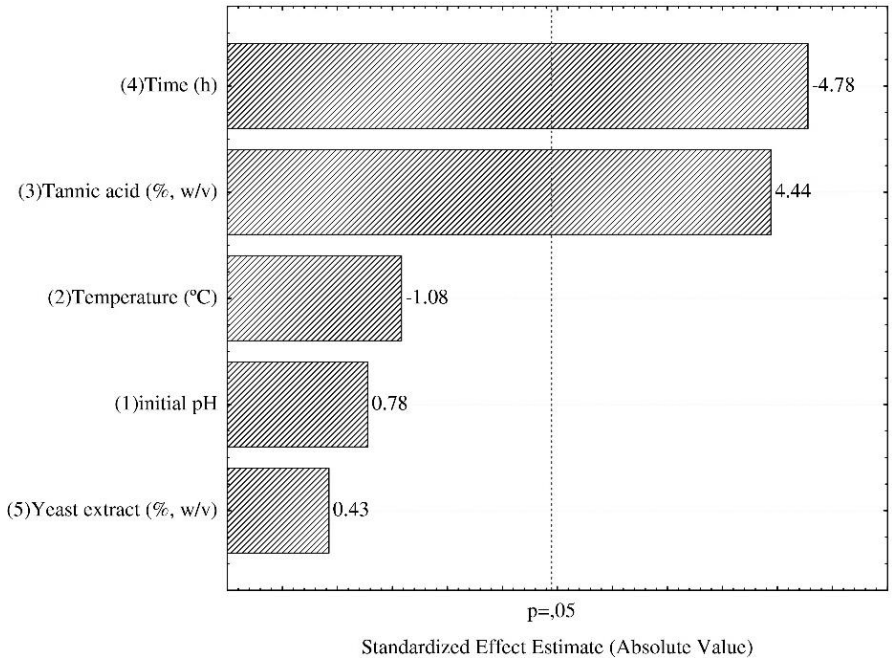

Figure 1. The effect and coefficient for tannase production presented by the variables used in the Plackett-Burman design (PB).

Table 4. Significant variables used in response surface methodology (RSM).

Independent variables	-1	-0.866	-0.5	0	0.5	0.866	1
Time (h)	22	-	34	46	58	-	70
Tannic acid (%, w/v)	-	6	-	7	-	8	-

variable and the corresponding coefficient are presented in Figure 5. The significant variables were time and tannic acid, which shows that the influence of these variables was higher on the parameters tested in the production of tannase from *A. carneus* URM 5577.

The fermentation time and tannic acid concentration have been identified as the most significant variables for producing tannase from *A. carneus* URM 5577 in SSF using the Plackett-Burman model (Figure 1). For *A. aureolus*, only tannic acid was significant for fermentation: The other variables did not influence tannase production. These variables were selected to be optimized using the central composite design (CCD) parameters provided by the statistical program (Table 4).

Data were subjected to analysis of variance (ANOVA), with the tannase production levels and experimental data shown in Table 4. The quadratic regression equation better explained the optimization of environmental variables to produce tannase, with an R^2 of 0.98, explaining 98% of the variability of the model and showing its quality.

The interactions between variables generated three-dimensional graphs showing increased production of tannase, suggesting an optimal production (5571.09 U/gds) in culture medium with 7% tannic acid added and incubated at 28°C. Therefore, there was an increase of approximately 4% in the production of the enzyme, when compared with the maximum activity obtained in PB planning (5449.31 U/gds) and approximately 500% compared with the initial screening (1128.59 U/gds), proving the validity of the optimization model. According to the RSM planning, while simultaneously analyzing the factors time and tannic acid concentration, it was observed that the production of tannase from *A. carneus* URM 5577 increases according to the increase in tannic acid content and incubation temperature (Figure 2).

For this study, PEG of different molecular weights (3350, 6000, and 8000) were tested at pH 6, 7, and 8, and random points were chosen in the two-phase region according to the phase in which the tannase is set, in which case was the PEG. Tables 5 to 7 show the results obtained for the purification factor (PF), total activity (Y), and partition coefficient (K).

The effects of PEG concentration, potassium citrate, pH, and concentration of PEG on the purification factor are shown in Figure 4.

All variables showed significant effects. AMPEG was the independent variable that most influenced the

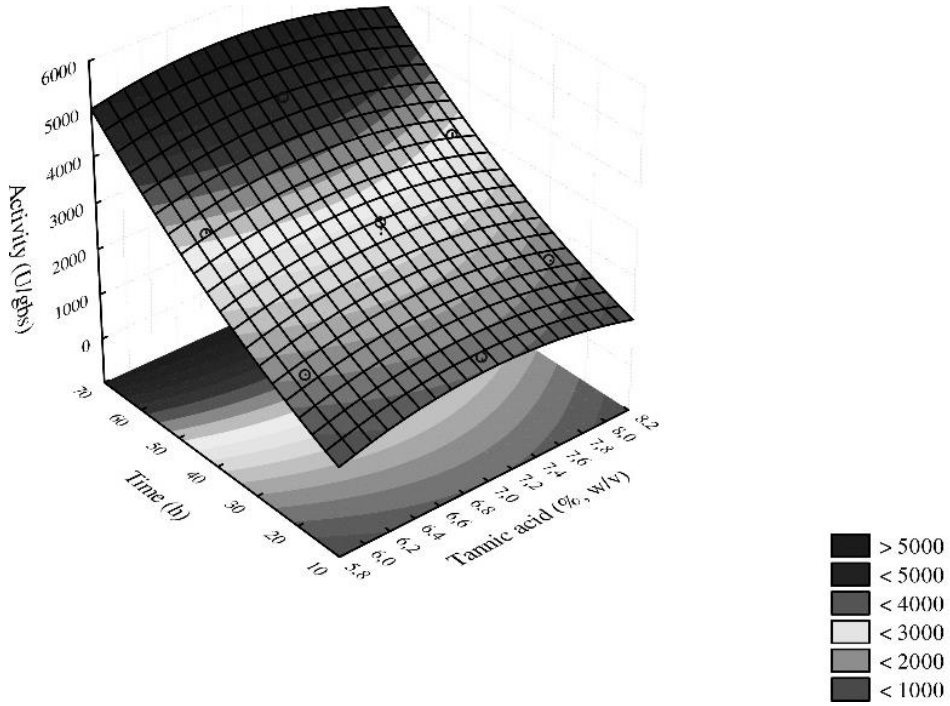

Figure 2. Response surface for the activity of tannase considering tannic acid and time.

Table 5. Tannase activity by ATPS based on PEG 3350 pH 6 and 8.

Run	PEG (%)	pH	Citrate potassium	PF	K	Y (%)
1	15	6	15	3.72	23.14	2166.38
3	25	6	15	2.13	17.84	1981.65
5	15	8	15	2.21	14.44	1280.49
7	25	8	15	1.36	16.29	2122.61
9	15	6	25	3.82	15.62	1941.95
11	25	6	25	3.58	21.82	2269.33
13	15	8	25	1.56	25.51	1749.58
15	25	8	25	2.44	21.18	1941.43

Table 6. Tannase activity by ATPS based on PEG 6000 pH 7.

Run	PEG %	pH	Citrate potassium	PF	K	Y (%)
17	20	7	20	1.93	7.89	1440.41
18	20	7	20	1.77	9.01	1382.69

response variables (K, Y, and FP). The interaction among the variables of the molecular weight of PEG, the pH of potassium citrate with the molecular weight of PEG and the concentration of PEG, and between pH and the concentration of PEG had significant positive effects: When levels of both variables were increased, the purification factor increased (Figure 3). pH will adversely interact with citrate, and PEG concentration, interacting with other variables, will also have a negative effect, namely, when the others are increased, the concentration of PEG will decrease.

In the application, the crude enzymatic extract of *A. carneus* URM 5577 containing tannase is shown in Figures 5 to 8. The pure mangaba juice presented a

Table 7. Tannase activity by ATPS based on PEG 8000 pH 6 and 8.

Run	PEG %	pH	Citrate potassium	PF	K	Y (%)
2	15	6	15	1.83	8.14	1906.95
4	25	6	15	1.73	10.58	599.64
6	15	8	15	2.75	11.44	1518.05
8	25	8	15	8.07	12.60	2327.76
10	15	6	25	4.95	10.35	1366.20
12	25	6	25	6.42	14.22	1514.90
14	15	8	25	8.15	17.03	1389.52
16	25	8	25	7.39	18.90	2229.30

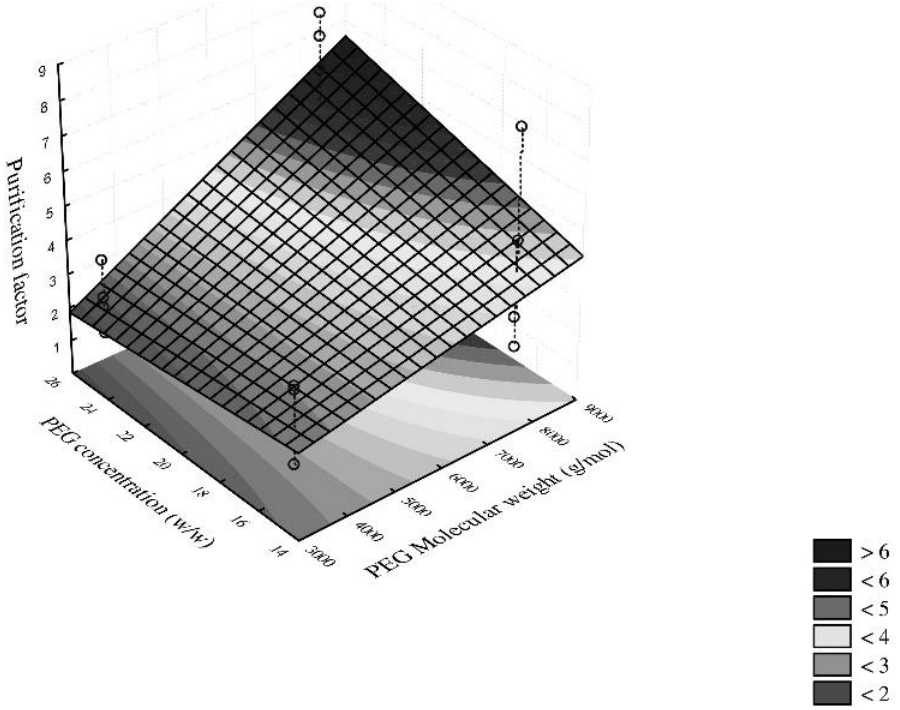

Figure 3. Response surface for the purification factor considering CPEG and MMPEG.

tannin concentration of 126.21 U/mL. Clarification was tested in different crude extract concentrations (Figure 5) and over different time intervals (Figure 6). These data were subjected to regression analysis, polynomial equations of the second degree were selected with an R^2 = 0.94 and statistical analysis was performed. A further increase in the volume of crude enzyme extract (2 mL) improved the point of clarification, since according to the prediction equation of the data, the maximum point was with a volume of crude extract of 63.53 U/mL tannase (Figure 6), whereas the minimum quantity of tannin showed no significant difference when applied to different concentrations of crude extract (Figure 5). The tannin content in the juice was reduced to 49.66% (63.53 U/mL) after 90 min of incubation with the crude enzyme extract in 2 ml at 37°C.

Pure tamarind juice contained 161.21 U/ml tannins (Figure 7) and underwent the same conditions of analysis as mangaba juice: R^2 = 0.99 for time and R^2 = 0.92 for extraction concentration. It also showed a further increase in clarification according to the volume of crude enzyme extract (2 ml), where its peak was at a volume of crude extract containing 77.67 U/ml tannase (Figure 8). The tannin content in the juice was reduced to 51.82% (77.67 U/mL) after 120 min of incubation with the crude enzyme extract in 2 ml at 37°C.

DISCUSSION

SSF has been proven to be of enormous benefit to the production of tannase by numerous authors (Gupta et al.,

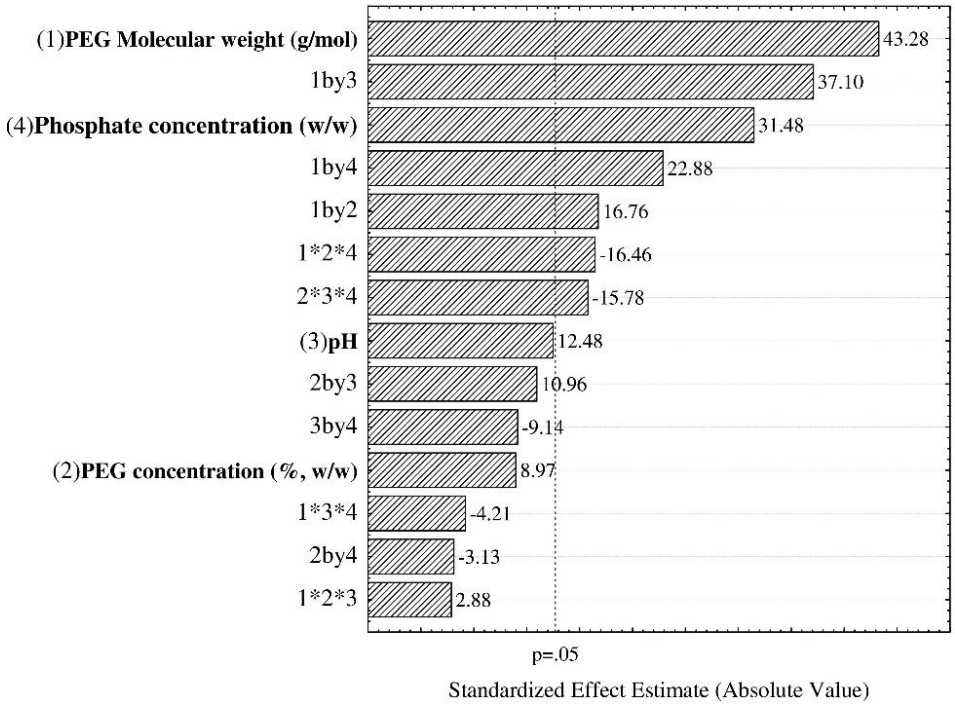

Figure 4. Comparison chart of the effects of purification factors.

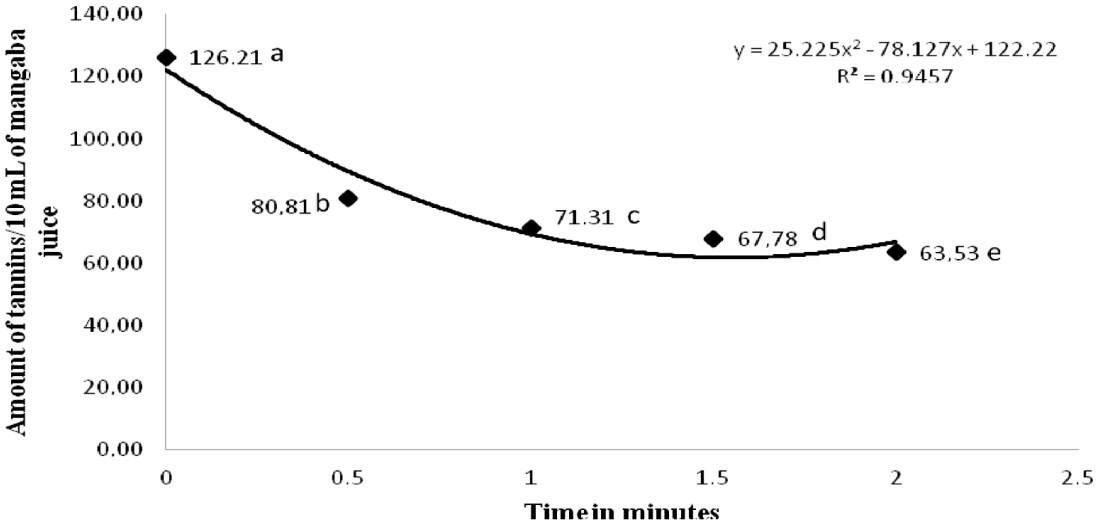

Figure 5. Effect of different volumes of crude extract on the degradation of tannins in mangaba juice after 90 min incubation.

2008; Wang et al., 2013) due to several factors, such as the low cost of energy and water and the reuse of sheets as a substrate (Selwal and Selwal, 2012).

In Brazil, especially in coastal regions, castanets (*T. catappa* L.) easily adapts to conditions of high salinity and strong winds (Thomson and Evans, 2006). Its leaves are rich in tannins, thus presenting an excellent carbon source for the production of tannase by SSF.

The possibility of using of agricultural waste as a source of carbon to produce enzymes by fungi has aroused the interest of scientists (Sabu et al., 2005). Selwal et al. (2011) used different leaves as substrates, such as amla (*Phyllanthus emblica*), ber (*Ziziphus mauritiana*), jamun (*Syzygium cumini*), Jamoa (*Eugenia cuspidate*), and keekar (*Acacia nilotica*) in SSF. In this study, the authors obtained a maximum activity of 170.75

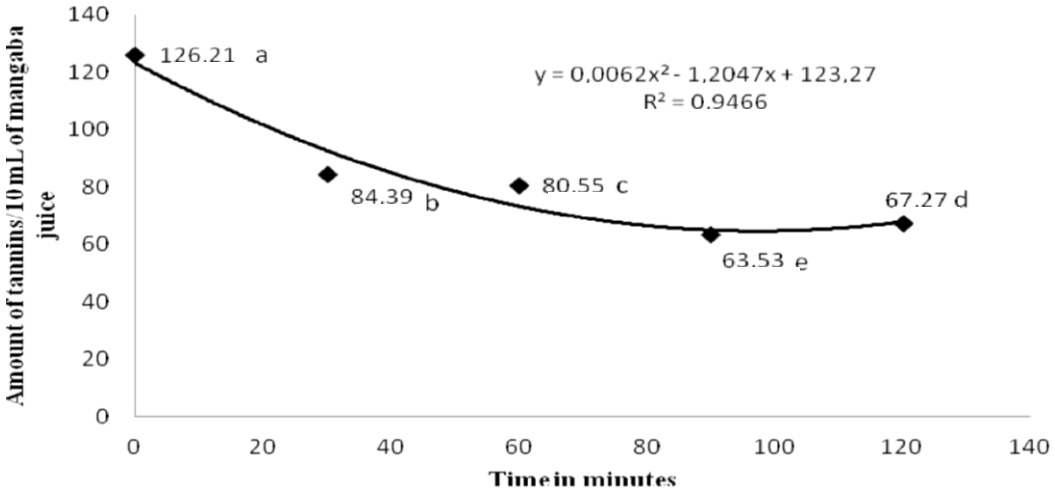

Figure 6. Effect of 2 ml of crude extract on the degradation of tannins from the juice mangaba at different times.

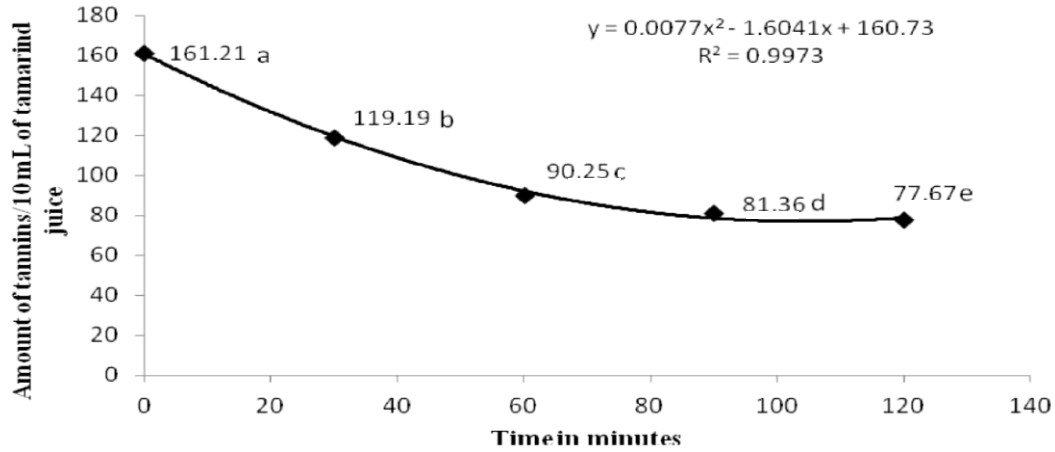

Figure 7. Effect of different volumes of crude extract on the degradation of tannins present in tamarind juice after 90 min incubation.

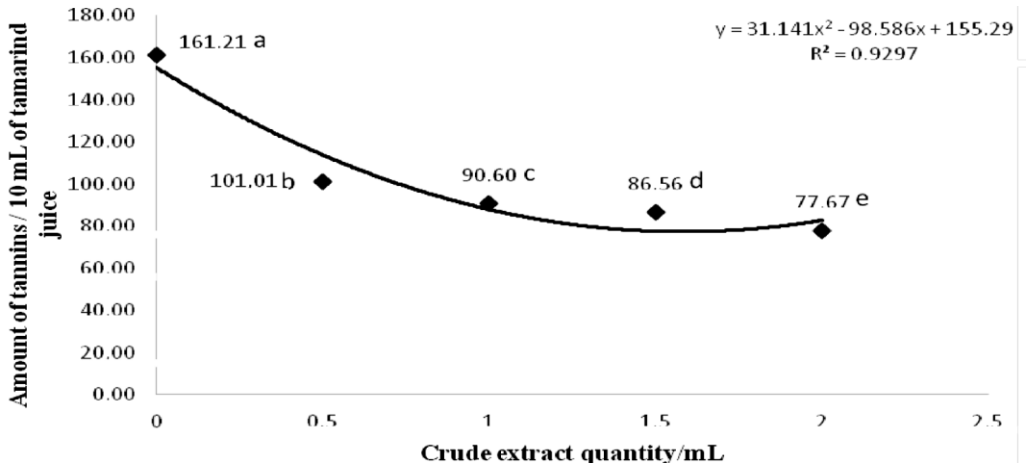

Figure 8. Effect of 2 ml of crude extract on the degradation of tannins present in tamarind juice at different times.

and 165.56 U/gds in jamun and keekar, respectively, after 96 h at 28°C. Macedo and Madeira Jr. (2012) evaluated the production tannase using orange pulp as a substrate by SSF using *Paecilomyces variotii* as biocatalyst. In this study, after optimization, an enzymatic activity of 5,000 U/gds was obtained using 3% tannic acid for 96 h.

The "castanhola" leaves used in this study produced significant results when compared with the literature, potentially increasing their activities by almost 5 times after optimization, with an activity of *A. carneus* URM5577 of 5571.09 U/gds. This is the first report of tannase production in this species. Thus, it is proven that the use of agro-forestry waste as a substrate for filamentous fungi is effective for producing tannase and is of great industrial interest for its potential to minimize the costs of enzyme production.

pH and time are very important variables in the production of metabolites (Selwal and Selwal, 2012). In this study, the greatest activity was shown at pH 6.0 for the 70 h shorter fermentation time. Shorter incubation times will be of interest to the enzyme production industry, as this will decrease the cost of production. Therefore, tannase production by *A. carneus* URM 5577 in a relatively short time is feasible and has proven itself highly efficient.

After applying PB design, time and percentage of tannic acid were identified as significant variables. According to PB, the shorter fermentation (48 h) enabled increased activity. However, after application of RSM, it was observed that the optimum time is 70 h for producing tannase by *A. carneus* URM 5577. In addition, RSM showed that the optimal concentration of tannic acid is 7%. Anwar (2007) obtained a better optimization of tannase production with 7% tannic acid by *A. niger*.

The purification factor showed significant results when compared with the literature. Rodríguez-Durán et al. (2011) used different concentrations of PEG 400, 600 and 1000 with potassium phosphate to optimize the purification of tannase produced by *A. niger* and obtained a recovery of 96%, which was recovered from in the lower phase of the system consisting of PEG 1000, and increased the purification factor by 7-fold.

All test results exceeding 100% may have been due to enzyme activation analysis, for example, upon removal of secondary metabolites during purification, enzyme activity could be inhibited, or otherwise increasing the concentration of salt and/or protein, which helps to maintain the conformation of the protein in its active form (Pan et al., 2001).

Evaluating the partition coefficient (K), it was observed that all K values were greater than one (1), demonstrating that the enzyme exhibits different partition trends between phases. Marcos et al., (1999) reported that the salt-rich phase (bottom phase) has hydrophilic characteristics, and the PEG-rich phase (top phase) has hydrophobic characteristics. Thus, partitioning of the enzyme showed a greater affinity for the PEG-rich phase (top phase), favored by a hydrophobic interaction in the system.

The juices from some fruits may have some undesirable characteristics, such as astringency, darkening, and bitter taste due to the presence of tannins in these fruits. The use of enzymatic treatment is growing in industries due to its low cost and high efficiency (Aguilar et al., 2007).

Sharma et al. (2014) observed the production of tannase by SSF in *A. niger* in the treatment of guava juice (*Psidium guajava*), where there was a reduction of 59.23% in tannin content after 60 min using 2% crude enzyme extract.

In another study, Lima et al. (2014) tested the activity of grape juice by using *Penicillium montanense* URM 6486, and found that it was more efficient at reducing tannin content by 46% after 120 min of incubation at 37°C and 2 ml of the crude extract. This study showed that the enzyme produced by *A. carneus* URM 5577 contained in 2 ml of the crude enzyme extract when applied to both juices showed efficiency when compared to the literature, thus demonstrating the relevance of fungal tannase in the clarification of juices rich in tannins.

Conclusions

A. carneus URM5577 is described for the first time to produce tannase by SSF using as a substrate "castanhola" leaves. The aqueous, two-phase system is a promising and cost-effective alternative in the purification of these enzymes. However, optimization of the growth parameters in a bioreactor is essential for the commercial viability of tannase and its possible application in the clarification of juices such as tamarind and mangaba.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Common bean germplasm resistant to races 73 and 2047 of *Colletotrichum lindemuthianum*

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The present work aims to identify common bean accessions resistant to anthracnose by incompatibility reaction evaluations to races 73 and 2047 of *C. lindemuthianum*, associated with using of $SF10_{1072}$ and $SAS13_{950}$ molecular markers linked, respectively, to gene *Co-10* and allele *Co-4²*. A total of 75 common bean accessions from Common Bean Germplasm Bank (CBGB) of Núcleo de Pesquisa Aplicada à Agricultura (Nupagri) were evaluated. The results showed that 14 accessions were resistant to both races 73 and 2047 of *C. lindemuthianum*, 36 exhibited resistances only to race 2047, and 25 revealed incompatibility reaction to race 73. Joint analysis between incompatibility phenotypic reactions and the presence of markers allowed identifying 13 resistant accessions to races 73 and 21 to race 2047, indicating the occurrence of *Co-10* (renamed *Co-3⁴*) and *Co-4²*, respectively. A total of 32 accessions were resistant without the presence of markers. Among the 75 common bean accessions evaluated, important sources of resistance to *C. lindemuthianum*, from Andean and Mesoamerican regions, were identified and have promising potential in breeding programs.

Key words: *Phaseolus vulgaris*, anthracnose, molecular marker, sequence characterized amplified regions for amplification of specific band (SCAR).

INTRODUCTION

Anthracnose is one of the most important common bean diseases (*Phaseolus vulgaris* L.) worldwide, causing crop yield loss up to 100% in regions with prevailing high humidity and moderate temperatures (13 to 27°C) (Vieira and Paula Júnior, 2004; Mendéz-Vigo et al., 2005). This disease is usually problematic in tropical and subtropical regions, where common bean is used as main protein source for human feed (Mendéz-Vigo et al., 2005), being considered endemic in Africa, Asia, Australia, and some other Latin American countries, such as Brazil (Vieira and

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Paula Júnior, 2004; Mahuku and Riascos, 2004).

Colletotrichum lindemuthianum (Sacc. & Magnus) Scrib. the causal agent of anthracnose in common bean, is an imperfect fungus characterized by its high pathogenic variability, which has approximately 120 races identified in the world (Mahuku and Riascos, 2004; Hernández-Godinez et al., 1998; Ferreira et al., 2008). In Brazil, it has already been identified in more than 50 races of *C. lindemuthianum* (Alzate-Marin and Sartorato, 2004; Damasceno and Silva et al., 2007; Gonçalves-Vidigal et al., 2008a; Sansigolo et al., 2008), considering that 64, 65, 73, 81, 87 and 89 pathotypes are the most frequent ones, mainly found in States of Paraná, Santa Catarina, Goiás and Distrito Federal.

The high pathogenic variability revealed by *C. lindemuthianum*, which has not been completely elucidated, is attributed to its parasexual cycle (Rodríguez-Guerra et al., 2004). The pathogen's reproduction mechanism is responsible for continuous resistance enhancement, mainly observed in commercial cultivars, since most of them present monogenic resistance that is easily overcame by emerging races (Rodríguez-Guerra et al., 2003). However, among the diverse strategies adopted to control anthracnose, genetic resistance is considered the most efficient and economical (Mahuku et al., 2002). For that, common bean genetic breeding programs in Brazil and worldwide, through the effort of many researchers, have been constantly seeking for new sources of genetic resistance to this pathogen.

Previous studies have identified 19 dominant anthracnose resistance genes. Some of these genes were found on Mesoamerican beans while other genes were discovered on Andean beans. The Mesoamerican genes include: *Co-2*, *Co-3* (and its alleles *Co-3*^{2}, *Co-3*^{3}, *Co-3*^{4}, *Co-3*^{5}), *Co-4* (and its alleles *Co-4*^{2}, *Co-4*^{3}), *Co-5* (and its allele *Co-5*^{2}), *Co-6*, *Co-10* (renamed as *Co-3*^{4}; Gonçalves-Vidigal et al., 2013), *Co-11*, *Co-15*, Co-*16*, *Co-17*, *Co-u*, and *Co-v* and the Andean genes Co-*1* (and its alleles *Co-1*^{2}, *Co-1*^{3}, *Co-1*^{4}, *Co-1*^{5}), *Co-12*, *Co-13*, *Co-14*, *Co-15*, *Co-x*, *Co-w*, *Co-y*, and *Co*-z (Geffroy, 1997; Geffroy et al., 1999, 2008; Kelly and Vallejo, 2004; Gonçalves-Vidigal and Kelly, 2006; Gonçalves-Vidigal et al., 2007, 2008b, 2009, 2011, 2013; Sousa et al., 2014, 2015; Trabanco et al., 2015; Coimbra-Gonçalves et al., 2016). Among these genes, *Co-10* (renamed as *Co-3*^{4}) possesses ample resistance spectrum and is considered incompatible to 17 races of *C. lindemuthianum* normally found in Brazil regions, especially race 73 (Alzate-Marin et al., 2003; Costa et al., 2003).

On the other hand, allele *Co-4*^{2} is considered the most effective to control this disease, showing a resistance index of 97% when tested with 34 races of *C. lindemuthianum* (Balardin and Kelly, 1998), pointing out of them race 2047. This race was firstly described by Mahuku and Riascos (2004), in isolates from Costa Rica, and it is considered one of the most virulent ones because it is capable of overcoming the anthracnose's resistance conferred by seven genes (*Co-1, Co-2, Co-3, Co-4, Co-5, Co-6* and *Co-11*) and five alleles (*Co-1*^{2}*, Co-1*^{3}*, Co-1*^{5}*, Co-3*^{3} e *Co-4*^{3}).

In addition to gene identification by conventional methods, the use of molecular markers to characterize resistant genes to anthracnose has significantly contributed for initial steps of breeding programs by reducing the time and costs involved during the whole process. This occurs because DNA markers are closed linked to genes and are not influenced by environmental factors and they show epistatic or minimum/none pleitropic effects (Agarwal et al., 2008).

Among the available molecular markers, the ones denominated as SCAR (Sequence characterized amplified regions for amplification of specific band) have been playing great importance on common bean analyses. Until now, there are 14 SCAR markers linked to anthracnose resistance genes, which are: SE_{ACT}/M_{CCA}, $SCAreoli_{1000}$, $SQ4_{1440}$, $SW12_{700}$, $SY20_{830}$, $SC08_{910}$, $SAS13_{950}$, $SH18_{1100}$, $SBB14_{1150/1050}$, $SAB3_{400}$, $SZ20_{845}$, $SZ04_{567}$, $SB12_{350}$ and $SF10_{1072}$ [21]. SCAR markers have been optimized in breeding programs that search for anthracnose resistant cultivars by implanting assisted backcrosses programs (Miklas and Kelly, 2002), during characterization of accessions in the beginning of the selection process or to obtain superior lineages (Beraldo et al., 2009).

Therefore, the present work's objective was to identify common bean accessions resistant to anthracnose through evaluations of phenotypic reactions to races 73 and 2047 of *C. lindemuthianum* associated to the using of $SF10_{1072}$ and $SAS13_{950}$ molecular markers linked to *Co-10* and *Co-4*^{2}, respectively.

MATERIALS AND METHODS

Plant material

Identification of resistance sources to races 73 and 2047 of *C. lindemuthianum* was carried out by evaluation of 75 common bean accessions from CBGB of Nupagri, Universidade Estadual de Maringá (Table 1).

Among the 75 studied accessions, 27 were collected in Mato Grosso do Sul State and kindly provided by Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Centro Nacional de Pesquisa de Arroz e Feijão (CNPAF) according to Material Transference Deal – II (MTD-II). A total of 18 accessions from active germplasm bank were given by Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (Epagri), which were collected in Santa Catarina State. The accessions BGF1 to BGF 20 were collected in the region of Toledo in Paraná State by the agronomist Rodrigo Garcia and donated to the active common bean germplasm bank of Nupagri, whereas the other 13 accessions used in this study already belong to Nupagri's germplasm bank.

Seeds from each accession were sown in polyethylene vases (48 × 30 × 11 cm) containing a mixture of previously fertilized and sterilized substrate. The plant vases were kept in a greenhouse until the first trifoliolate leaves (stage V3) were fully expanded. At that time, the plants were inoculated with race 73 and 2047 of

Table 1. Registration number of CBGB/Nupagri (RN), common name, gene pools Andean (A) and Mesoamerican (M) and origin from all 75 common bean accessions evaluated.

RN		Common name	Gene pool	Origin
1	BGF1	-	M	Parana
2	BGF2	-	A	Parana
3	BGF3	-	A	Parana
4	BGF4	-	M	Parana
5	BGF5	-	M	Parana
6	BGF6	-	A	Parana
7	BGF9	Corinthiano	A	Parana
8	BGF11	-	A	Parana
9	BGF12	-	M	Parana
10	BGF13	Pixirum	M	Parana
11	BGF14	-	A	Parana
12	BGF15	Pitanga	A	Parana
13	BGF16	-	A	Parana
14	BGF17	-	A	Parana
15	BGF18	Paco Parana	M	Parana
16	BGF19	-	A	Parana
17	BGF20	-	A	Parana
18	BGF28	Carioca Pintado I	M	Parana
19	BGF29	Carioca Pintado II	M	Parana
20	BGF30	Carioca Pitoko	M	Parana
21	BGF31	Iapar 31	M	Parana
22	BGF35	Preto IV	M	Parana
23	BGF36	Rosinha	M	Parana
24	BGF37	Roxinho	A	Parana
25	BGF39	Jalo Pardo	M	Parana
26	BGF40	Jalo Pintado I	A	Parana
27	BGF41	Jalo Pintado II	A	Parana
28	BGF44	Rosinha Opaco	M	Mato Grosso do Sul
29	BGF45	Rosinha A	M	Mato Grosso do Sul
30	BGF46	Rosinha B	M	Mato Grosso do Sul
31	BGF47	Rosinha C	M	Mato Grosso do Sul
32	BGF48	Rosinha D	M	Mato Grosso do Sul
33	BGF49	Roxinho A	M	Mato Grosso do Sul
34	BGF50	Carioquinha Limpo	M	Mato Grosso do Sul
35	BGF51	Rosado	A	Mato Grosso do Sul
36	BGF52	Mulatão Lustroso	M	Mato Grosso do Sul
37	BGF53	Bico de Ouro A	M	Mato Grosso do Sul
38	BGF54	Bico de Ouro B	M	Mato Grosso do Sul
39	BGF55	Mulatinho Vagem Roxa A	M	Mato Grosso do Sul
40	BGF56	Mulatinho Vagem Roxa B	M	Mato Grosso do Sul
41	BGF57	Carioca Vagem Rosada	M	Mato Grosso do Sul
42	BGF58	Jalo	A	Mato Grosso do Sul
43	BGF60	Uberabinha Preto	M	Mato Grosso do Sul
44	BGF61	Manteiguinha de Cipó	A	Mato Grosso do Sul
45	BGF62	Jalo sem Cipó	A	Mato Grosso do Sul
46	BGF63	Carioca sem Cipó	M	Mato Grosso do Sul
47	BGF64	Bodoquena	A	Mato Grosso do Sul
48	BGF66	Rosinha sem Cipó	M	Mato Grosso do Sul
49	BGF68	Manteiga com Cipó	A	Mato Grosso do Sul
50	BGF70	Bolinha	A	Mato Grosso do Sul

Table 1. Contd.

51	BGF71	Roxinho Mineiro	M	Mato Grosso do Sul
52	BGF72	Preto Guamirim	M	Mato Grosso do Sul
53	BGF74	Rosinha Guaicurus	M	Mato Grosso do Sul
54	BGF75	Cara Suja	M	Mato Grosso do Sul
55	BGF76	Rio Tibagí [S-89-N(1)] -Introduzido do IICA	M	Costa Rica
56	BGF77	Aporé	M	Embrapa
57	BGF78	Iapar 44	M	Iapar
58	BGF91	Preto Brilhoso Achatado	A	Santa Catarina
59	BGF92	Preto Precoce Cunha Porã	M	Santa Catarina
60	BGF93	Crioulo Manteiga	A	Santa Catarina
61	BGF94	Crioulo Brilhoso	M	Santa Catarina
62	BGF95	Crioulo 159	M	Santa Catarina
63	BGF96	CF 75	A	Santa Catarina
64	BGF97	FC 2016	A	Santa Catarina
65	BGF98	FC 2001	A	Santa Catarina
66	BGF99	FC 2045	A	Santa Catarina
67	BGF100	Crioulo Brilhoso Ponte Serrada	M	Santa Catarina
68	BGF103	FC 89	M	Santa Catarina
69	BGF104	Preto Redondo	A	Santa Catarina
70	BGF105	FC 117	M	Santa Catarina
71	BGF110	Azulão Ponte Serrada	A	Santa Catarina
72	BGF111	Azulão Ab. Luz	A	Santa Catarina
73	BGF112	Porto Real	M	Santa Catarina
74	BGF113	Safira	M	Santa Catarina
75	BGF114	Amendoim Cavalo	A	Santa Catarina

C. lindemuthianum and this procedure was repeated for two times. Seedlings were grown under natural light in greenhouses supplemented by 400 w high-pressure sodium lamps giving total light intensity of 115 µmoles m^{-2} s^{-1} for 7 to 10 days until they reached the first trifoliate leaf stage. Subsequently, inoculations with races 73 and 2047 of *C. lindemuthianum* were carried out and analyzed. Finally, we conducted molecular analysis through SCAR $SF10_{1072}$ and $SAS13_{950}$ markers.

Preparation of samples for DNA extraction

Ten seeds of each accession were grown in plastic trays containing peat moss and vermiculite, and kept in greenhouse until they reached the first trifoliate leaf stage. After this period, a young leaflet of each seedling was individually collected with 1.5 mL plastic micro tubes frozen in liquid nitrogen and stored in freezer (-20°C) for DNA extraction. Subsequently, the trays were transferred to mist chamber with controlled temperature (22 ± 2°C), and later they were inoculated with spores suspension of races 73 and 2047 of *C. lindemuthianum*.

Inoculum preparation and virulence characterization

Inoculum was prepared according to the methodology proposed by Cárdenas et al. (1964), which consists on multiplying spores of each *C. lindemuthianum* pathotype in test tubes containing sterilized pods partially immersed in agar-water (AW) culture media. After inoculation, test tubes were incubated for 14 days at 20 ± 2ºC. After incubation period, pods from each test tube were removed using tweezers and transferred to a Beaker containing sterile distilled water. Double gauze was used to filter the suspensions obtained for each pathotype, and the spore suspension was adjusted to 1.2 x 10^6 spores mL^{-1} through dilutions with distilled sterile water. Each pathogen was inoculated separately using small brushes (Tigre® model 266, number 14). After inoculation, the plants were placed in a mist chamber for 48 h at a temperature of 20 ± 2°C with light controlled at 12 h of daylight and 12 h of darkness (light intensity of 300 micromoles m^{-2} s^{-1} at a height of 1 m) and a relative humidity of 95%. After the incubation period, the inoculated plants were transferred to open-air benches at a temperature of 22°C with artificial light (12 h of daylight at 25°C), where they remained for 3 days before visual symptom assessment. Anthracnose disease reactions were rated visually using a scale from 1 to 9 (Pastor-Corrales et al., 1995). The plants scored from 1 to 3 were considered resistant, whereas the ones scored from as 4 to 9 were susceptible.

Genomic DNA extraction and analysis using SCAR markers

DNA extraction was carried out based on methodology proposed by Afanador et al. (1993). All amplification reactions were performed with a thermal cycler (MJ Research Inc., Waltham, MA). The polymerase chain reaction (PCR) program for $SF10_{1072}$ consisted of 3 min at 94°C, 35 cycles of 15 s at 94°C, 1 min at 65°C, and 90 s at 72°C, followed by a 7 min extension at 72°C and 4 min at 4°C (Corrêa et al., 2000). PCRs were performed in 25 µL total reaction volumes containing 40 ng total DNA; 0.2 mM each dNTP; standard

Table 2. Joint analysis of 75 common bean accessions, considering reactions to races 73 and 2047 of *C. lindemuthianum* and the presence (+) or absence (-) of markers $SF10_{1072}$ and $SAS13_{950}$.

Common bean reaction	73/$SF10_{1072}$	2047/$SAS13_{950}$
Resistant with the presence of marker (R^+)	BGF5, BGF12, BGF13, BGF46, BGF49, BGF52, BGF55, BGF56, BGF58, BGF68, BGF70, BGF78, BGF95	BGF1, BGF3, BGF6, BGF9, BGF11, BGF15, BGF20, BGF41, BGF44, BGF50, BGF62, BGF66, BGF70, BGF71 BGF93, BGF96, BGF98, BGF99, BGF100, BGF104, BGF111
Resistant without the marker (R^-)	BGF4, BGF6, BGF11, BGF15, BGF16, BGF17, BGF19, BGF20, BGF29, BGF37, BGF41, BGF51, BGF61, BGF77, BGF100, BGF105, BGF114	BGF5, BGF18, BGF37, BGF45, BGF51, BGF52, BGF68, BGF74, BGF94, BGF95, BGF97, BGF110, BGF112, BGF113, BGF114
Total of resistant accessions	30	36
Susceptible with the presence of marker (S^+)	BGF36, BGF44, BGF48, BGF53, BGF54, BGF57, BGF60, BGF63, BGF64, BGF66, BGF71, BGF72, BGF74, BGF75, BGF76	BGF4, BGF12, BGF13, BGF14, BGF16, BGF17, BGF19, BGF31, BGF39, BGF40, BGF46, BGF48, BGF53, BGF54, BGF55, BGF56, BGF58, BGF60, BGF61, BGF76, BGF77, BGF78, BGF91, BGF103
Susceptible without the marker (S^-)	BGF1, BGF2, BGF3, BGF9, BGF14, BGF18, BGF28, BGF30, BGF31, BGF35, BGF39, BGF40, BGF45, BGF47, BGF50, BGF62, BGF91, BGF92, BGF93, BGF94, BGF96, BGF97, BGF98, BGF99, BGF103, BGF104, BGF110, BGF111, BGF112, BGF113	BGF2, BGF28, BGF29, BGF30, BGF35, BGF36, BGF47, BGF49, BGF57, BGF63, BGF64, BGF72, BGF75, BGF92, BGF105
Total of susceptible accessions	45	39

Taq buffer containing 1.5 mM $MgCl_2$ and 0.2 µM forward primer and reverse primer; and 1 unit of *Taq* DNA polymerase. Following the addition of 2 µL loading buffer (30% glycerol and 0.25% bromophenol blue), The PCR products from SF10 were visualized on agarose gels. Amplification reactions consisted in a total volume of 25 µL each, were conducted for $SAS13_{950}$ as proposed by Young et al. (1998). The PCR products from $SAS13_{950}$ were fractionated in agarose gel 1.2% prepared with TAE 1X buffer (40mM Tris-acetate, 20 mM acetic acid and 1mM EDTA), containing ethidium bromide (0.02%). The DNA bands were visualized under ultraviolet light, and digital images were recorded with an L-PIX Image EX model (Loccus Biotecnologia - Loccus do Brasil, Cotia, SP, Brazil). Gel evaluations were conducted to identify accessions with the presence of 1072 bp and 950 bp bands, which correspond to markers SF10 and SAS13, respectively.

Phenotypic data and SCAR markers were analyzed as follow: resistant plants with marker (R^+), resistant plant without marker (R^-), susceptible plants with marker (S^+) and susceptible plant without marker (S^-).

RESULTS AND DISCUSSION

Incompatibility reaction of common bean accessions to races 73 and 2047 of *C. lindemuthianum*

Table 2 shows the phenotypic reactions to races 73 and 2047 of *C. lindemuthianum*. Based on the results, it was observed that out of the 75 analyzed accessions 30 of them were resistant to race 73, whereas 36 were to race 2047. It was also noted that 14 accessions showed resistance to both races.

Among the 30 accessions resistant to race 73, 15 are Mesoamerican and 15 are Andean. The Andean accessions from Manteigão commercial group prevailed with 12 resistant accessions (BGF 6, BGF 11, BGF 16, BGF 17, BGF 20, Pitanga, Jalo Pintado II, Jalo, Manteiguinha de cipó, Manteiguinha com cipó, Bolinha and Amendoim Cavalo), whereas among Mesoamerican commercial groups Preto (BGF 4, Iapar 44, Crioulo 159, Crioulo Brilhoso Ponte Serrada and FC 117) and Roxinho (BGF 12, Roxinho and Roxinho A) revealed greater importance. The other accessions belong to commercial groups of Rosinha (BGF 5 and Rosinha B, Pixirum and Rosado), Carioca (Carioca Pintado II and Aporé), Mulatinho (Mulatinho Vagem Roxa A and Mulatinho Vagem Roxa B),) and Pardo (Mulatão Lustroso). It is important to mention that 14 accessions are from Paraná, 10 from Mato Grosso do Sul, four from Santa Catarina and two are commercial cultivars (Aporé and Iapar 44).

In the incompatibility reaction to race 2047, the analysis verified that 26 accessions belong to Andean gene pool, while 10 belong to the Mesoamerican. Originally, 14 from these accessions were collected in Santa Catarina, 11 in Parana State and the others in Mato Grosso do Sul. The commercial group with greater number of resistant accessions was Preto (BGF 3, Crioulo Manteiga, Crioulo Brilhoso, Crioulo 159, CF 75, FC 2016, FC 2001, FC 2045, Crioulo Brilhoso Ponte Serrada, Preto Redondo CN694 FC 1212, Azulão Ponte Serrada and Azulão Ab. Luz), from which 11 accessions are from Santa Catarina of the Epagri's common bean breeding program. The second group with greater number of accessions with resistance was Manteigão (BGF 6, BGF 11, BGF 20, Pitanga, Jalo Pintado II, Jalo sem Cipó, Manteiga com Cipó, Bolinha and Amendoim Cavalo).

An important observation obtained from this study is

that out of the 75 analyzed accessions, 14 of them were resistant to both races 73 and 2047 of *C. lindemuthianum*. Besides that, 10 of them are Andean and belong to commercial group Manteigão (BGF 6, BGF 11, BGF 20, Pitanga, Jalo Pintado II, Manteiga com cipó, Bolinha and Amendoim Cavalo), group Rosinha (Rosado) and group Roxinho (Roxinho). On the other hand, four accessions are Mesoamerican and are allocated in the commercial groups of Preto (Crioulo 159 and Crioulo Brilhoso Ponte Serrada), Rosinha (BGF 5) and Pardo (Mulatão Lustroso).

A total of 75 accessions were analyzed, from which 44 are Mesoamerican and 31 Andean. According to the results obtained, when inoculated with race 73, 42% of Andean accessions and 38.6% of Mesoamerican accessions were resistant. On the other hand, when inoculation was carried out with race 2047, 61% of Andean accessions and 38.6% of Mesoamerican accessions demonstrated incompatibility to this race. It is important to point out that both races 73 and 2047 are Mesoamerican. Melotto and Kelly (2000) have affirmed that Andean accessions tend to be more resistant than Mesoamerican isolates, a fact that was evident when the evaluated accessions were tested with race 2047.

Common bean was independently domesticated from weedy wild beans in two separate geographic centers, the Mesoamerican, extending from Mexico to Colombia; and the Andean, extending from Colombia to Argentina (Gepts and Debouck, 1991). Correspondingly, two genes pools known as the Mesoamerican and the Andean have been described for common bean. Beans from the Mesoamerican gene pool are small to medium-seeded and contain significantly wider genetic diversity than the mostly large-seeded Andean beans (Beebe et al., 2000; 2001; Chacon et al., 2005). Similarly, studies of the virulence and genetic diversity of *C. lindemuthianum* using differential cultivars and molecular techniques reveal that the diversity of *C. lindemuthianum*, as well as that of the rust (*Uromyces appendiculatus*) and angular leaf spot (*Phaeoisariopsis griseola*) pathogens, segregates into two distinct groups that mirror the diversity of their common bean host (Guzman et al., 1995). These two groups have been also named Andean and Mesoamerican because they correspond to the Andean and Mesoamerican gene pools of their common bean host. Andean races of the anthracnose pathogen are usually isolated from large-seeded beans that belong to the Andean gene pool. Conversely, Mesoamerican races are often, but not always, isolated from small or medium-seeded beans belonging to the Mesoamerican gene pool. Mesoamerican races of this pathogen exhibit considerably greater virulence and genetic diversity than the Andean races. More importantly, Andean races of this pathogen are compatible only or mostly with the Andean beans, while Mesoamerican races are compatible with Mesoamerican and Andean beans, but are generally more virulent on the former. Thus, when breeding beans with anthracnose resistance to Mesoamerican races of *C. lindemuthianum*, Andean anthracnose resistance loci are extremely valuable. The Andean *Co-1* resistance loci have been very valuable in breeding Mesoamerican beans with anthracnose resistance, particularly in production countries where Mesoamerican races of the anthracnose pathogen predominate (Kelly and Vallejo, 2004). Conversely, when controlling Andean races, anthracnose resistance loci from Mesoamerican beans are very important. It has been posited that the Andean and Mesoamerican races of *C. lindemuthianum* have evolved separately; Andean races with Andean beans in South America and Mesoamerican races with Mesoamerican beans, respectively. The previous discussion highlights the need to identify and characterize additional resistant genes in Andean beans to complement the many anthracnose resistance genes available in Mesoamerican common beans.

Joint analysis between incompatibility reaction to races 73 and 2047 and SCAR molecular markers

Joint analysis between incompatibility reaction and the presence of molecular markers $SF10_{1072}$ and $SAS13_{950}$ are shown in Table 2.

Incompatibility reaction of accessions to race 73 and data regarding $SF10_{1072}$ marker revealed that 17.3% of resistant accessions showed the presence of the marker linked to gene *Co-10* (renamed *Co-3*^{4}). Similarly, $SAS13_{950}$ marker was efficient to identify 28% of accessions resistant to race 2047. The other accessions that exhibited a marker, either linked to gene *Co-3*^{4} or *Co-4*^{2} allele, were considered susceptible to races 73 and 2047. In addition, no marker was detected in 22.7 and 20% of accessions considered resistant to races 73 and 2047, respectively.

The molecular analysis revealed that the resistant accessions, in which amplification of marker was noted, possess gene *Co-3*^{4} and/or *Co-4*^{2} allele. Therefore, it was observed the occurrence of resistant accessions did not present the molecular marker might had happened due to resistance breaking between gene and marker by recombination process (Melotto et al., 2004). This fact could indicate that these 29 accessions may have different resistance gene(s) from those evaluated in this study.

As noted already by Melotto and Kelly (2001), the fact that susceptible accessions have amplified the markers might have occurred due to intragenic recombination. These authors have demonstrated intragenic recombination in *Co-4* locus, which resulted in susceptible recombinants capable to amplify the marker even at a lower rate. The *Co-4* locus contains several paralogs of the *COK-4* gene that is predicted to code for a receptor-like kinase (RLK). The predicted *COK-4* protein is highly similar to *FERONIA* (*FER*), a membrane-

localized protein of Arabidopsis, as they cluster in a single clade of the RLK phylogenetic tree (Melotto and Kelly, 2001; Melotto et al., 2004; Oblessuc et al., 2015). Receptor-like kinases (RLKs) are important pattern recognition receptor (PRRs) that play a role in self and non-self-recognition, including the perception of hormones (Shiu and Bleecker, 2001), pathogen associated molecular patterns (PAMPs) and pathogen effectors.

Joint analysis between incompatibility reactions to races 73 and 2047 and molecular markers pointed out that many accessions possess at least one Mesoamerican resistance gene, as: *Co-4*, *Co-5*, *Co-6*, *Co-10* and *Co-4*^{2}, or another gene not yet identified. Indeed, these accessions will have to be analyzed through allelism tests to determine the independence of the anthracnose resistance gene in these accessions from the known anthracnose resistance genes in common bean. Additionally, wide-genome association using Single Nucleotide Polymorphism (SNP) assay and Kompetitive Allele Specific PCR (KASP) markers will be conducted for genotyping these accessions and mapping resistance genes to *C. lindemuthianum*.

Common bean accessions analyzed in this study have shown incompatibility to two pathotypes that consist of important anthracnose resistance sources, especially those that present resistance to race 2047. Among the 75 common bean accessions that belong to Nupagri's Germplasm Bank were identified important resistance sources to *C. lindemuthianum* of Andean and Mesoamerican origins that could be available for breeding programs. In addition, the identification of resistant accessions without association to analyzed markers points out that they could represent new resistance sources to anthracnose. Therefore, genetic characterization of these accessions is essential for their future using in breeding programs.

Conclusions

Among the 75 common bean accessions evaluated, important sources of resistance to *C. lindemuthianum*, from Andean and Mesoamerican regions, were identified and have potential in breeding programs.

Furthermore, the identification of accessions resistant to *C. lindemuthianum* by the markers present new perspectives for the use of molecular marker assisted selection, making it more agile and effective selection process in breeding programs.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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