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Full Length Research Paper

Screening endophytic actinobacteria with potential antifungal activity against *Bipolaris sorokiniana* and growth promotion of wheat seedlings

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Actinobacteria secrete substances that limit or inhibit the growth of plant pathogenic fungi and may be used in the biocontrol of these microorganisms. The aim of this study was to characterize physiological and enzymatic activity of endophytic actinobacteria, evaluate their antifungal activity against *Bipolaris sorokiniana* root colonization, and evaluate their efficiency in promoting the growth of wheat seedlings. Antibiosis was analyzed using the double-layer method, the agar well diffusion test, and volatile metabolites. Physiological and enzymatic activity was evaluated through chitinase, glucanase, siderophores, indole-3-acetic acid, nitrogen fixation and phosphate solubilization tests. I*n vivo* assays were evaluated by root colonization, biocontrol test and efficiency to promote the growth of wheat seedlings. From all isolates tested, 69.6% of them presented antifungal activity against at least one *B. sorokiriana* isolate. Among these, 17% of the isolates produced bioactive metabolites in the supernatant when grown in submerging culture. The highest production of bioactive metabolites was at 30°C, between 72 and 96 h of incubation. Three isolates produced volatile compounds, chitinase, glucanase, siderophores and exhibited nitrogen fixation, produced indole-3-acetic acid, efficiently colonized the root system of seedlings of two wheat cultivars. The best isolate [6(2)] showed, under the greenhouse, the capacity to promote an increased biomass and tillers per wheat plant.

Key words: Antagonist actinobacteria, antifungal activity, biocontrol, spot blotch, lytic enzymes.

INTRODUCTION

The genus, *Bipolaris* includes some significant plant pathogens with worldwide distribution. A common root rot and leaf spot caused by *Bipolaris sorokiniana* are important diseases, causing a large amount of damage, to wheat and barley crop in warm and humid regions of the world (Duveiller et al., 2005). The losses due to foliar blight can vary among the regions. Some studies report that the losses of a wheat plantation may be as high as

100% under very severe conditions of infection (Metha, 1978). In favorable clime conditions, the disease occurs throughout the culture cycle.

Traditionally, chemical controls have been recommended to prevent the losses caused by the pathogen, to treat both seeds and to fight the disease already established in the plantation (Reis et al., 2005). A sustainable agriculture requires a plant-disease control that is more ecological and less dependent on synthetic chemical products. Biological control has been considered as a safe method for controlling soil-borne pathogens (Ling et al., 2010). The biological control maintains the balance in agricultural ecosystems, protecting hosts against significant damage caused by phytopathogens (Júnior et al., 2000).

Actinobacteria have been indicated as potential biocontrol agents against plant pathogens diseases (Igarashi, 2004; Pal and McSpadden Gardener, 2006; El-Tarabily et al., 2010; Costa et al., 2013; Zhang et al., 2014). These microorganisms play a major role in the rhizosphere by secreting a broad range of antimicrobial products that prevent the growth of common root pathogens (Khamna et al., 2009; Oliveira et al., 2010; Sobolevskaya and Kuznetsova, 2010). Besides their potential in biological control, their traits in plant growth promoting is already known (Jog et al., 2012; Nimaichand et al., 2013; Gopalakrishnan et al., 2014). El-Tarabily et al. (2010) observed enhanced plant growth and yield with the application of endophytic *Actinoplanes campanulatus, Micromonospora chalcea* and *Streptomyces spiralis,* individually and in combination, on cucumber seedlings. Costa et al. (2013) evaluating the control of *Pythium aphanidermatum* in cucumber (*Cucumis sativa* L.) under greenhouse conditions, observed that endophytic isolate both of *Streptomyces* genus, were able to reduce damping-off incidence.

Their mechanisms of action involve parasitism of hyphae and lytic enzymes, competition with pathogens, production of antibiotics, siderophores and pesticides (Crawford et al., 1993; Igarashi, 2004; Hasegawa et al., 2006; El-Tarabily and Sivasithamparam, 2006; Khamna et al., 2009; Gangwar et al., 2014).

The metabolic perspective of actinomycetes not only provides an attractive area for research but also offers the possibility of commercialization of the metabolites generated in the process (Sharma, 2014). The worldwide efforts in search of natural products for the protection market have progressed significantly, and Actinobacteria, especially genus *Streptomyces*, appear to be good candidates in finding new approaches to control plant diseases. Several commercial products derived from actinobacteria are available for use in crop protection and growth promotion (Palaniyandi et al., 2013; Hamdali et al., 2008; Minute et al., 2006).

In this scenario, the present study aimed to characterize and evaluate endophytic actinobactéria isolates for their ability to suppress the phytopathogen *B. sorokiniana* based on the production of secondary metabolites and the wheat seedlings root colonization.

MATERIALS AND METHODS

Microorganisms

The assays started with twenty-three actinobacteria obtained from tomato roots (*Lycopersicon esculentum*) (Oliveira et al., 2010) and twenty-two *B. sorokiniana* isolates from different Brazilian regions provided by EMBRAPA – Passo Fundo, RS, Brazil. All biological material used in this study belongs to the collection of the Environmental Microbiology Laboratory, DMPI, ICBS, UFRGS, RS, Brazil.

Antifungal activity

The antifungal activity of 23 actinobacterial isolates was assessed using 22 *B. sorokiniana* isolates. Actinobacteria were inoculated using the spot method in starch casein agar (SCA) medium (10 g starch, 0.3 g casein, 2.0 g KNO_3, 2.0 g NaCl, 2.0 g K_2HPO_4, 0.05 g $MgSO_4.7H_2O$, 0.02 g $CaCO_3$, 0.01 g $FeSO_4.7H_2O$, 15.0 g agar, distilled water to complete 1 L) and incubated at 28°C for seven days. The double-layer agar method was used; 10 mL of melted potato dextrose agar (PDA) and inoculated with a *B. sorokiniana* suspension (10^6 spores/mL), poured on actinobacteria colonies and incubated at 28°C for four days. The essay was carried out in triplicate. The formed antibiosis halos were measured, and the antifungal activity (IA) was determined by the mean differences of the halo diameter and colony diameter.

Production of antifungal compounds in submerged culture

The actinobacteria that exhibit the wider spectrum activity in the double-layer assay were chosen to optimize growth conditions and metabolites production in submerged culture. The isolates were inoculated in 250 mL conic flasks containing 50 mL starch casein broth (SCB) and incubated at 20, 25, 28, 30 and 40°C for 48 h under agitation (115 rpm). From this culture, an aliquot of 10% (v/v) was transferred to new flasks containing 50 mL of SCB and incubated under the same conditions as described for seven days. Then, 1 mL was retrieved from each flask every 24 h, transferred to microtubes and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a new tube and used in the antifungal activity assay.

Antifungal activity of the supernatant

The antimicrobial activity of the isolates was determined by the agar diffusion method (Bauer et al., 1966). The actinobacteria which showed inhibitory halo equal or greater than 1.0 cm, in the double-layer assay, were grown under submerged conditions at 20, 25, 28 and 30°C. The antimicrobial activity of the supernatant was tested against five *B. sorokiniana* isolates (98004, 98012, 98032, 98040

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and 98041). These isolates were selected based on previous results (Minotto et al., 2014). Wells were punctured, using a sterile cork borer, in PDA plates previously seeded with one of the five *B. sorokiana* chosen. One hundred microliters (100 µL) of the supernatant of each isolate was added to each well. Incubation took place at 4°C for 18 to 20 h for diffusion of the bioactive compound into the medium. After that, incubation of plates took place at 28°C for four days in the dark. Following incubation, inhibition zones were measured. These assays were carried out in triplicate.

Pairing of cultures and volatile compounds

Actinobacteria isolates were tested against *B. sorokiniana* using the pairing of cultures in dishes containing PDA and with overlapping dishes (Dennis and Webster, 1971). The pairing of cultures was carried out to analyze the direct antagonistic action of actinobacteria against *B. sorokiniana*. The overlapping dishes technique was used to observe the production of volatile compounds (VCOs) by the actinobacteria, which may influence the growth of phytopathogen (Hutchinson, 1967). Dishes were inoculated for each test and incubated at 28°C in the dark. The pairing of cultures was evaluated after seven days of incubation. The antagonistic activity was determined as the distance between the edges of the antagonist colony and the phytopathogen. VCOs production was recorded after 192 and 336 h of incubation considering the growth of fungal mycelia. Fungal growth inhibition (%) was calculated using the formula (*R1 – R2*/*R1*) x 100, where *R1* is the radial growth of the fungus with no exposure to actinobacteria, and *R2* is the radial growth of the fungus inoculated with the actinobacteria. These experiments were carried out in triplicate.

Enzymatic and physiological characterization

The 23 actinobacterial isolates were tested for their capacity to hydrolyze chitinase and β-1,3-glucanase, solubilize phosphate, siderophores production, indole-3-acetic acid (IAA) and fixing nitrogen. All assays were carried out at 25, 28 and 30°C with an exception for IAA production, which was determined only at 28°C. Enzymatic and physiological assays were conducted in triplicate, and the data obtained were analyzed using the analysis of variance and the Tukey's test (α = 0.05) using the software SASM-Agri (Canteri et al., 2001).

Production of chitinase and β-1,3-glucanase

Each of the isolates was spot-seeded on a mineral salt agar medium containing 0.5% laminarin (Sigma L9634), or 0.08% colloidal chitin to detect β-1,3-glucanases or chitinases (Renwick et al., 1991), respectively. The colloidal chitin was prepared according to El-Dein et al. (2010). After 14 days of incubation, hydrolysis zone around colonies were measured and chitinolytic and β-1,3-glucanases activity was determined. Enzymatic index (AI) was determined following Rosato et al. (1981).

Phosphate solubilization

The phosphate solubilization assay was carried out as described by Nautiyal (1999). Isolates were inoculated on plates containing NBRIP solid medium (National Botanical Research Institute's phosphate growth medium devoid of yeast extract) using the spot method and incubated for 21 days. The evaluation was determined based on the presence (phosphate solubilizers) or absence of halos under the colony growth.

Production of siderophores

The production of siderophores was assessed as previously described by Schwyn and Neilands (1987). Isolates were inoculated using the spot method on trypticase soy agar (TSA) ten times diluted and supplemented with chrome-azurol S complex ([CAS/iron (III)/hexadecyl trimethyl ammonium bromide] and incubated for 14 days. The positive reaction shows an orange or yellow halo around the colonies.

Production of indole-3-acetic acid (IAA)

Production of IAA follows the methodology described by Gordon and Weber (1951). Isolates were inoculated in TSA supplemented with 10% tryptophan 5 mM and incubated at 28°C for 14 days under agitation of 100 rpm. Then cultures were centrifuged, and 2 mL of the supernatant was transferred to test tubes containing 1 mL of Salkowski reagent (Gordon and Weber, 1951). The mixture was incubated at 28°C for 30 min in the dark. The evaluation was carried out in a spectrometer at 530 nm. AIA concentration ($mg.L^{-1}$) was determined to prepare a calibration curve with different concentrations of synthetic IAA (0, 1, 2, 3, 6, 10 and 16 $\mu g\ L^{-1}$).

Nitrogen fixation

The assay for nitrogen-fixing was performed as described by Döbereiner et al. (1995). Actinobacteria were spot-inoculated on test tube containing NFb semisolid medium and the culture was incubated at 25, 28 and 30°C for 14 days. A positive reaction was indicated by presence of veil inside the growth culture medium

***In vitro* colonization of wheat seedling roots by actinobacteria isolates**

Wheat seedlings roots colonization by the actinobacteria was assessed as described by Queiroz et al. (2006) with adaptations. Wheat seeds of BRS Buriti and BRS Camboatá cultivars were surface disinfected and then submitted to microbiolization. The seeds were immersed in separate suspensions containing propagules of actinobacteria and incubated under agitation for 4 h at 25°C. Suspensions were prepared growing microorganisms in SCA medium for 10 days at 28°C. The bacterial propagules concentration was adjusted to A_{540} = 0.50 nm.

After microbiolization, two seeds of each wheat cultivar were transferred to tubes containing agar-water (0.8%) and incubated at 25°C with 12 h photoperiod for seven days. Wheat seedlings roots colonization by actinobacteria was assessed as described by Queiroz et al. (2006). Roots were sliced into 2 to 3 cm fragments long and placed in plates containing SCA and PDA media and plates incubated for seven days at 28°C to confirm the presence of actinobacteria in the rhizosphere. The experimental design was totally randomized with five repetitions, with four actinobacterial isolates, which showed the best results in all assays, and wheat cultivars (BRS Buriti and BRS Camboatá). Seeds immersed only in saline were the negative control.

***In vivo* assay**

The *in vivo* experiment was conducted under natural sunlight and temperature and receiving different conditions of the weather. The

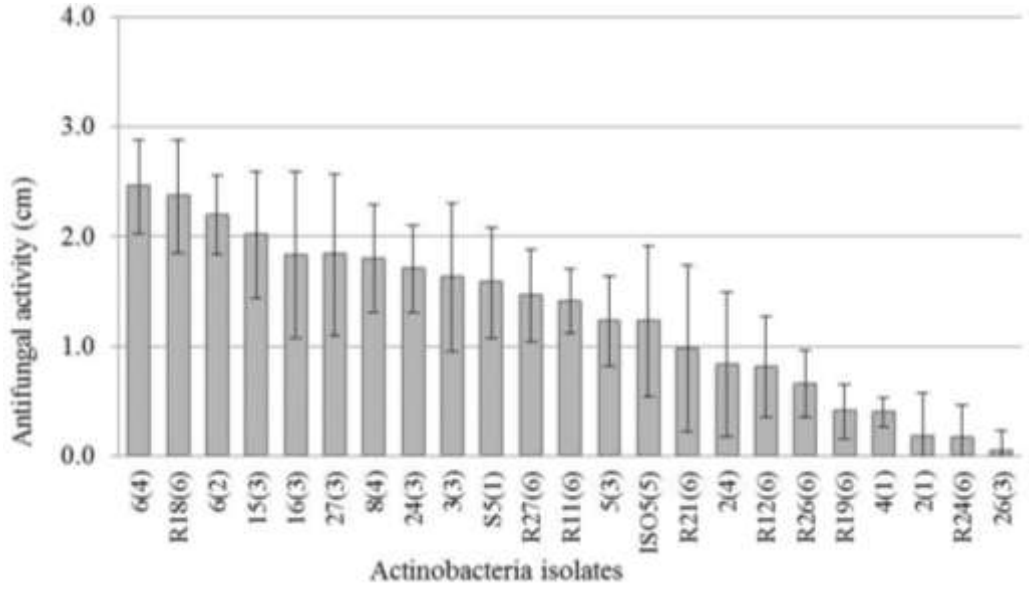

Figure 1. Antifungal activity (cm) of 23 endophytic actinobacteria against 22 *Bipolaris sorokiniana* strains isolated from different Brazilian regions, using the double-layer method.

assay was performed in pots containing sterile vermiculite and sand (2:1). In each pot, were placed five wheat seeds of BRS Buriti or BRS Camboatá cultivars. After germination, thinning was performed leaving three seedlings per pot.

For this study, eight treatments were determined: (1) Seeds immersed in SCB / soil without infestations (control); (2) Microbiolized seeds with actinomycetes/soil without infestations (control); (3) Microbiolized seeds with actinomycetes/soil infested with *B. sorokiniana*; (4) Untreated seeds/soil infested with actinomycetes; (5) Untreated seeds/soil infested with *B. sorokiniana*; (6) Microbiolized seeds with actinomycetes/sprayed *B. sorokiniana*; (7) Microbiolized seeds with actinomycetes/soil infested *B. sorokiniana* in time of plantation; (8): untreated seeds/soil infested with *B. sorokiniana* at plantation time;

Treatments 7 and 8 soil was infected with a pathogen suspension (5×10^5 spores/mL in the proportion of 0.5 g/mL of the substrate) at the time of planting. In treatments 3 and 5, the soil infestation occurred at the stage GS 15, 23 (Zadoks et al., 1974). In treatment 4, a suspension of 10^8 CFU/mL antagonists was added to the soil at the planting of the seeds. In treatments 4 and 6, the aerial part of the wheat plants was sprayed with *B. sorokiniana* (5×10^5 spores/mL) using a manual spray with a distance of 40 cm from the sheet. The infected plants were at the stage GS 15, 23 (Zadoks et al., 1974). The plants were subjected to the humid chamber for 24 h before and 48 h after spraying.

The experiment took place from June 13 to 2 September 2011. The period was characterized by high rainfall (172.3 mm), 26% above the average of the three previous years for the same period (BDMEP, 2015). The average of temperature was between 10.6 and 19.4°C, relative humidity average between 79 and 84% and the average monthly insolation period was 109.13 h (BDMEP, 2015). The plants were watered as necessary and received a nutritive solution (Voss and Scheeren, 2006) three times a week. The evaluation of leaf necrosis was performed at the fourth true leaf in the 7, 14, 21 and 28 days after inoculation. The assessment based on the rating scale infection response (IR) described by Fetch Jr and Steffenson (1999) was used. Also, the numbers of tillers per plant, fresh mass weight, dry biomass weight and shoot height. The experiment consisted of eight treatments for each of the two cultivars, five replicates per treatment were evaluated with three observational units.

RESULTS

Antifungal activity

All actinobacterial isolate inhibited, at least one *B. sorokiniana* isolate. Of the 23 actinobacteria tested, 65.2% (15) presented antifungal activity against *B. sorokiniana* with values greater than 1.0 cm. The isolates 6(4), R18(6), 6(2),15(3) presented the highest inhibition zone against fungi growth (2.45, 2.37, 2.20 and 2.01 cm, respectively) (Figure 1), which represents growth inhibition of 86.4, 77.3, 86.4 and 59.1% of *B. sorokiniana* isolates, respectively.

Production of antifungal compounds in submerged culture

The results obtained in submerged culture shows that isolates 6(2), 6(4) and 16(3) produced the highest amounts of active metabolites against *B. sorokiniana* at 30°C with 72 h of growth (Figure 2A, B and C, respectively). Active metabolites were produced by isolate R18(6) at temperatures of 20 and 25°C with 168 and 72 h of incubation, respectively (Figure 2D). Although, microorganisms' growth was observed at all tested temperatures, the largest antifungal activity occurred between the 72 and 144 h of growth at 30°C (Figure 2).

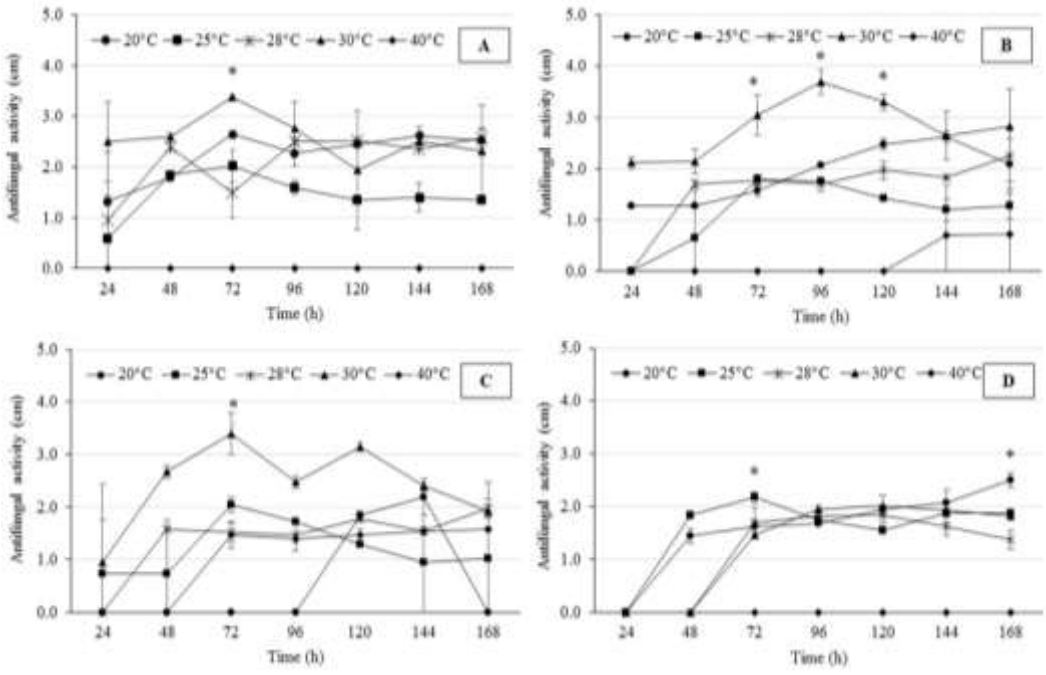

Figure 2. Production of antifungal compounds with inhibitory activity (cm) by actinobacteria in submerged culture at different incubation times and temperatures. A: Isolate 6(2); B: Isolate 6(4); C: Isolate 16(3); D: Isolate R18(6). *Statistically significant means in the Tukey's test (α = 0.05).

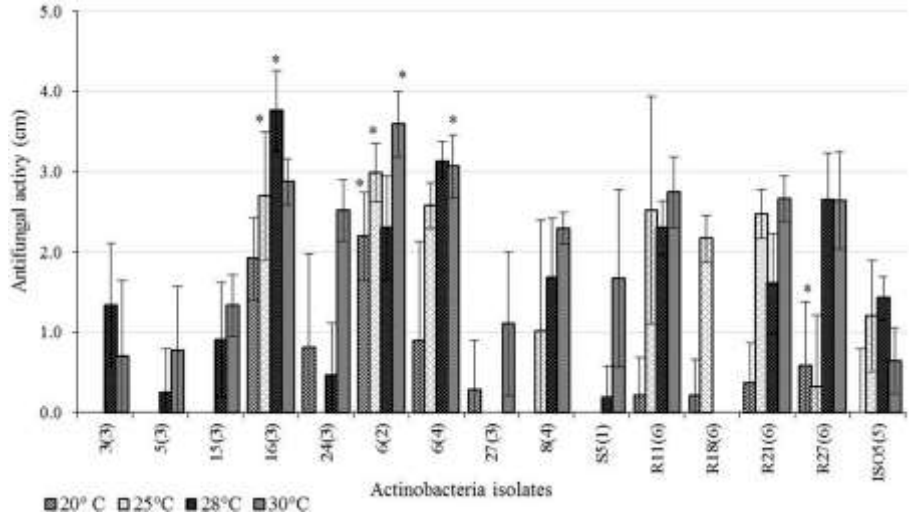

Figure 3. Antifungal activity (cm) against *B. sorokinana* isolates of the supernatant from the 15 actinobacteria, grown at different temperatures using the agar diffusion method. *Statistically significant means in the Tukey's test (α = 0.05).

Antifungal activity of the supernatant

The supernatant produced by 15 actinobacteria isolates showed antifungal activity against *B. sorokiniana* at least in one of the measured temperature (Figure 3). The highest number of isolates (93.3%) showed antifungal activity when grown at 30°C. However, isolate 6(2) showed antifungal activity with a statistical difference as compared to the results of the other isolates and temperatures. Isolate 16(3) showed the higher inhibitory activity as compared to all of them when grown at 28°C (Figure 3).

Table 1. Physiological and enzymatic activity (phosphate solubilization, siderophore production, nitrogen fixation, chitinase, β-1,3-glucanase and indole-3-acetic acid (AIA)) of actinobacteria isolates grown at different incubation temperatures.

Actinobacteria isolates	Phosphate solubilization			Siderophore production			Nitrogen fixation			Chitinase			Glucanase			AIA (Mean±SD) (µg L^{-1})
	25°C	28°C	30°C	25°C	28°C	30°C	25°C	28°C	30°C	25°C	28°C	30°C	25°C	28°C	30°C	28°C
6(2)	+	+	+	-	+++	++	-	-	+	2.0	2.0	2.1	2.8	2.5	2.3	33.21±8.75
3(3)	+	+	+	+++	+	-	-	+	+	16.0*	0.0	0.0	0.0	0.0	0.0	32.10±1.28
5(3)	-	-	+	+	+	+++	+	+	+	5.4	10.5	0.0	0.0	5.4	4.9	33.69±3.63
15(3)	-	+	+	-	++	+++	-	-	+	13.7	11.9	16.0*	0.0	5.0	4.7	32.09±1.50
16(3)	+	+	+	+	-	-	+	+	+	6.0	2.9	6.4	4.5*	3.8	3.4	32.09±1.50
24(3)	+	+	+	-	-	-	-	+	+	5.1	3.5	6.1	0.0	3.2	3.0	32.72±0.64
27(3)	-	+	+	++	-	-	-	-	-	6.0	3.1	6.3	0.0	2.9	2.6	33.59±4.49
6(4)	+	+	+	-	++	++	+	+	+	0.0	3.0	3.7	0.0	2.0	2.4	32.57±3.74*
8(4)	+	+	+	+++	+++	+++	+	+	+	11.0	0.0	0.0	0.0	0.0	0.0	32.79±1.07
S5(1)	+	+	+	-	++	+++	-	-	-	5.2	2.4	6.1	0.0	4.0	3.8	33.27±2.35
R11(6)	+	+	+	+++	-	++	-	-	+	8.0	0.0	2.1	0.0	0.0	0.0	32.75±1.28
R18(6)	-	-	-	-	++	-	-	+	-	1.1	1.1	2.3	2.0	1.2	0.9	33.77±1.67
R21(6)	+	+	+	-	+++	-	+	+	+	2.5	2.0	2.5	2.1	1.0	0.7	33.14±7.05
R27(6)	+	+	+	++	+++	++	-	-	-	4.5	1.6	1.8	0.0	1.4	1.3	32.50±2.99
ISO5(5)	+	+	+	-	+++	-	-	+	+	2.13	2.13	2.17	0.0	1.1	1.3	32.89±1.07

(-) Isolate with no activity; (+) Isolate with positive activity and siderophore production H/C ≤ 1; (++) Isolate with positive activity and H/C ≥ 1 ≤ 3; (+++) Isolate with positive activity and H/C > 3. (SD) Standard Deviation; (*) statistically significant means in the Tukey's test (α = 0.05).

Physiological and enzymatic activity

The enzymatic activity and physiological assays showed that isolates 6(2), 6(4) and 16(3) were able to solubilize phosphate, produce siderophore, fix atmospheric nitrogen and hydrolyze chitin and glucan. At 30°C, 14 isolates were positive for phosphate solubilization on solid medium. The isolate R18(6) did not solubilize phosphate at any of the measured temperature (Table 1). Siderophore production was observed in 93.3% of the isolates. Out of that 10 isolates, 66.6% produced an enzymatic index greater than 3.0. However, isolate 24(3) did not produce siderophores. With the increase in growth temperature from 25 to 30°C, there was an improvement in 40% of isolates in their capacity to fix nitrogen. Isolates 27(3) and S5(1) were unable in fixing nitrogen (Table 1).

Chitinase and β-1,3-glucanase production was observed for 100 and 80% of the isolates, respectively. For chitinase production, 26.6% of the isolates showed an enzymatic index greater than 10. The highest enzymatic index for chitinase was for isolates 3(3) and 15(3) at 25 and 30°C, respectively, and for glucanase was for isolate 16(3) at 25°C (Table 1). All actinobacteria incubated at 28°C for seven days synthesized auxins. IAA production oscillated between 6.7 µg L^{-1} for isolates 3(3) and 5(3) and 30.7 µg L^{-1} for isolate 6(4) (Table 1).

Pairing of cultures and volatile compounds

The results of the antibiosis assays carried out by direct comparison of two cultures show that isolates 6(2), 6(4) and 16(3) suppressed the development of five *B. sorokiniana* isolates (98004, 98012, 98032 and 98040). Antifungal activity was observed as the formation of an inhibition area ≥ 2.0 cm, between the edge of the

Table 2. Inhibition of *Bipolaris sorokiniana* isolates growth (%) by volatile compounds produced by actinobacteria.

Incubation time (h)	Inhibition of mycelial growth of *B. sorokiniana* (%) by actinobacteria isolates		
	6(2)	6(4)	16(3)
48	5.04	0.51	4.87
96	5.64	4.32	13.51
144	5.14	10.62	13.87
192	6.76	11.77	14.47
264	7.92	13.90	16.23
336	11.32	14.85	16.95

Table 3. Hypersensitivity reaction (IR) (extension of leaf necrosis) in wheat plants caused by *B. sorokiniana*, subjected to different treatments.

Treatment	Cultivar BRS Buriti				Cultivar BRS Camboatá			
	7days	14 days	21 days	28 days	7days	14 days	21 days	28 days
1	0	0	1	1	1	0	0	1
2	0	0	0	1	2	0	0	0
3	1	1	1	1	3	0	0	0
4	3	5	7	8	4	2	6	6
5	0	0	0	1	0	5	0	1
6	2	3	4	4	2	4	5	6
7	0	0	1	1	1	1	1	1
8	1	1	1	2	0	0	0	1

IRs 1, 2, and 3 low compatibility; IRs 4 and 5 intermediate compatibility.; IRs 6, 7, 8 and 9 high compatibility. (1) Seeds immersed in AC broth medium/ soil without infestations (control); (2) Microbiolized seeds with actinomycetes / soil without infestations (control); (3) Microbiolized seeds with actinomycetes/soil infested with *B. sorokiniana*; (4) Untreated Seeds/soil infested with actinomycetes; (5) Untreated seeds / soil infested with *B. sorokiniana*; (6) Microbiolized seeds with actinomycetes/Sprayed *B. sorokiniana*; (7) Microbiolized seeds with actinomycetes/soil infested *B. sorokiniana* in time of plantation; (8): untreated seeds/soil infested with *B. sorokiniana* at plantation time.

actinobacteria colony and the edge of the phytopathogen colony. The volatile compounds produced by the isolates showed decreased in the development of the fungal mycelium of *B. sorokiniana* with the increase of exposure time to the metabolites (Table 2). The reduction of *B. sorokiniana* growth was 4.16% in the first evaluation after 48 h of exposure, to 15.66% in the last assessment at 336 h of incubation.

Volatile compounds produced by isolates 6(2), 6(4) and 16(3) promoted the radial decrease in fungal mycelia (11.32, 14.85 and 16.95%, respectively), after 336 h of incubation (Table 2).

In vivo experimental assay

The results obtained in the *in vivo* assay showed that the extent of leaf necrosis in plants of treatments 4 and 6, which had shoots sprayed with *B. sorokiniana* showed small necrotic lesions (IRs 3), in the first assessment (seven days), and extensive leaf lesions with distinct chlorotic margins (IRs 6, 7 and 8) after the third evaluation (28 days), for both cultivars studied (Table 3). The other treatments showed only slight necrotic lesions without chlorosis or very light and diffuse marginal chlorosis (IRs 1, 2 and 3), considered to be indicative of low compatibility (Table 3).

The soil infestation with the isolate 6(2) (treatment 4) provided a significant increase in fresh weight (12.69 and 12.64 g) and dry weight (3.51 and 2.54 g) for both BRS Buriti and BRS Camboatá cultivars, respectively, as compared to the other treatments. This result showed that soil infestation with the antagonist promoted further development of wheat plants as compared to treatments with or without microbiolization (Table 4). Data analysis for plant height variable showed no significant difference between treatments for any of the cultivars (Table 4). Also, the soil infestation with isolate 6(2) significantly increased the mean number of tillers per plant for both cultivars as compared to treatment with or without seeds

Table 4. Fresh weight (g), dry weight (g) and average height (cm) of wheat plants submitted to different treatments, after 80 days of plantation.

Treatment	Cultivar BRS Buriti			Cultivar BRS Camboatá		
	Fresh weight (g)	Dry weight (g)	Height (cm)	Fresh weight (g)	Dry weight (g)	Height (cm)
1	9.98	2.40	48.80	10.02	2.19	37.40
2	10.46	2.45	45.00	9.64	2.08	38.00
3	8.74	2.09	45.00	9.38	1.93	37.80
4	12.67*	3.51*	48.60	12.64*	2.84*	40.00
5	9.56	2.19	47.80	10.88	2.43	41.80
6	9.87	2.26	47.20	8.50	1.70	39.20
7	9.59	2.19	48.40	10.10	2.15	38.80
8	8.90	2.12	47.00	8.85	1.80	38.20
C.V.	16.17 %	23.96 %	8.60 %	18.92 %	20.51%	7.82 %

(C.V.) Coefficient of variation. (1) Seeds immersed in AC broth medium/soil without infestations (control); (2) Microbiolized seeds with actinomycetes/soil without infestations (control); (3) Microbiolized seeds with actinomycetes/soil infested with *B. sorokiniana*; (4) Untreated Seeds/soil infested with actinomycetes; (5) Untreated seeds / soil infested with *B. sorokiniana*; (6) Microbiolized seeds with actinomycetes/Sprayed *B. sorokiniana*; (7) Microbiolized seeds with actinomycetes / soil infested *B. sorokiniana* in time of plantation; (8): untreated seeds / soil infested with *B. sorokiniana* at plantation time. *Statistically significant means in the Scott-Knott test (α = 0.05).

Table 5. Average number of tillers per plant wheat subjected to different treatments during the period of 80 days.

Treatment	Average number of tillers / plant					
	Cultivar BRS Buriti			Cultivar BRS Camboatá		
	7 days	21 days	28 days	7 days	21 days	28 days
1	1.13	1.20	2.07	1.53	2.03	2.60
2	0.73	2.07	2.20	1.53	2.13	2.27
3	0.73	0.73	1.80	1.20	1.67	3.13
4	2.33*	2.40*	2.80*	2.07*	2.47	3.07
5	0.73	1.40	2.07	1.73	1.93	2.67
6	0.60	1.33	2.60	0.67	2.33	3.53
7	0.47	1.13	1.47	1.20	1.93	2.27
8	0.87	1.20	1.93	1.07	2.13	2.93

(C.V.) Coefficient of variation. (1) Seeds immersed in AC broth medium/ soil without infestations (control); (2) Microbiolized seeds with actinomycetes/soil without infestations (control); (3) Microbiolized seeds with actinomycetes/soil infested with *B. sorokiniana*; (4) Untreated Seeds/soil infested with actinomycetes; (5) Untreated seeds/soil infested with *B. sorokiniana*; (6) Microbiolized seeds with actinomycetes/Sprayed *B. sorokiniana*; (7) Microbiolized seeds with actinomycetes / soil infested *B. sorokiniana* in time of plantation; (8): untreated seeds / soil infested with *B. sorokiniana* at plantation time. * Statistically significant means in the Scott-Knott test (α = 0.05).

microbiolized (Table 5). The number of tillers, subject to treatment 4, remained high throughout the evaluation period for cultivar BRS Buriti. For BRS Camboata cultivar, a significant difference was observed only in the first assessment (Table 5).

DISCUSSION

Actinobacteria populations are essential components of the endophytic and rhizospheric microbial community of several higher plant species. The production of antibiotics or other toxic metabolites by these microorganisms has been widely reported as a biocontrol tool against plant diseases (El-Tarabily et al., 2000; Cao et al., 2005; Castillo et al., 2006; Qin et al., 2011; Costa et al, 2013). Additionally, endophytic *Streptomyces* may improve the agricultural production, reducing the impact of root and crown rot (Coombs et al., 2004). Of the endophytic actinobacteria investigated in the present study, 69.6%

presented significant inhibitory activity against *B. sorokiniana* in the double-layer assay. Of these, isolates 6(2), 6(4) and 16(3) were able to maintain high antifungal activity when grown in submerged culture, as well as in the pairing of cultures in solid media. These results are higher than the findings reported in the literature for the inhibition of 6.5% of the fungi *Alternaria solani, B. sorokiniana, Fusarium oxysporum fsp. lycopersiccum, Gerlachia oryzae, Sclerotinia sclerotiorumaa, Verticillium alboatrum* and *Rhizoctonia* sp. with endophytic actinobacteria (Oliveira et al., 2010). In an antagonism screening, Costa et al. (2013) evaluated if the endophytic *Streptomyces* strains were able to inhibit the plant pathogenic fungi growth. The results show that *S. sclerotiorum, P. aphanidermatum, R. solani, Fusarium* sp. and *P. parasitica* were inhibited by 47.5, 55.0, 62.5, 77.5 and 90% of the isolates, respectively. Li et al. (2014) isolated an endophytic *Streptomyces* strain CNS-42 isolated from *Alisma orientale* and showed a potent effect against *F. oxysporum* f. sp. *cucumerinum* and a broad antimicrobial activity against bacteria, yeasts and other pathogenic fungi. The *in vivo* biocontrol assays showed a significant reduction in disease severity and plant shoot fresh weight and height increased greatly in plantlets treated with strain.

In a previous study, the largest inhibition zones in the growth of *C. sativus* by *Chaetomium globosum* was 6.3 mm in the pairing of cultures (Aggarwal et al., 2004). The authors discovered that the filtered cultures of this antagonist reduced the pathogen's growth by 19.6 to 100%. In the present work, the supernatant of actinobacteria inhibited the growth of *B. sorokiniana*, forming inhibition zones up to 3.7 cm in diameter. Temperature determines the optimal conditions of metabolite production in submerged cultures. It is known that the optimal growth temperature ranges are wide while optimal temperature for secondary metabolites production lies in a narrow interval, of 5 to 10°C (Iwai and Omura, 1982). In the production of streptomycin by *Streptomyces griseus*, an increase of 1°C leads to a drop of 80% in antibiotic production (Dunn, 1985). However, in our case, isolate 6(2) produced antifungal compounds against *B. sorokiniana* throughout the incubation period (168 h). This flexibility in antifungal activity has also been demonstrated by the presence of inhibition halos at temperatures of 20, 25, 28 and 30°C.

The conditions of secondary metabolites production depend on the actinobacterial isolate. Iwai and Omura (1982) study has shown that the mean production times of metabolites with antimicrobial action was between 120 and 240 h of incubation. In the present study, the highest production of bioactive compounds was at 30°C between 72 and 96 h of incubation, with inhibition zones between 3.4 and 4.1 cm in diameter. Salamoni et al. (2012) working with *Streptomyces* 1S observed the highest production of antimicrobial compounds between 48 and 120 h of incubation at 28°C. Bervanakis (2008) observed that the optimal production of secondary metabolites was after 240 h of incubation at 27°C.

The volatile compounds produced by *Streptomyces philanthi* RM-1-138 isolated from the rhizosphere soil of chili pepper suppressed the growth of the *Rhizoctonia solani, Pyricularia grisea, Bipolaris oryzae* and *Fusarium fujikuroi* (52.85 to 100%) (Boukaew et al., 2013). Different results observed in this study, where the reduction of radial growth of the pathogen was 11.32, 14.85 and 16.95% shown by isolate 6(2), 6(4) and 16(3) after 336 h of incubation, respectively. The evolution of volatile organic compounds by soil microorganisms has been associated with the promotion of plant growth (Ryu et al., 2003) and the induction of systemic resistance in cultures (Farag et al., 2006), growth inhibition (Fiddaman and Rossall, 1994) and germination of spores of pathogenic fungi (Mckee en Robinson, 1988).

Isolates 6(2), 6(4) and 16(3) were selected from a set of 23 actinobacteria because they presented high antifungal activity in all tests they were subjected to. Also, these actinobacterial isolates exhibited other properties often associated with biocontrol agents, such as the ability to hydrolyze chitin and glucan, to produce siderophores and IAA, solubilize phosphate, fix nitrogen and colonize the rhizosphere of wheat seedlings. Furthermore, these isolates are efficient producers of several hydrolases enzymes, (Minotto et al. 2014). Studies have shown that the action of *Streptomyces* includes the inhibition of pathogens by the production of antifungal compounds (El-Tarabily and Sivasithamparam 2006), the competition for iron in siderophore production, and the production of degradation enzymes, like chitinase and glucanase (El-Tarabily et al., 2000).

Among the 15 actinobacteria, 26.6% presented high enzyme index for the two enzymes chitinase and glucanase (EI >10.0 and EI >4.0, respectively) (Table 1). A previous study has shown that EI higher than 1.0 indicates the secretion of enzymes with biotechnological potential (Fungaro et al., 2002). Also, *Streptomyces* isolates prescribed for the control of white rot in coal and for the growth promotion of plants have been shown to produce significant amounts of chitinase and of β-1,3-glucanase (Gopalakrishnan et al., 2013). Additionally, the production of chitinase and of glucanase was the main mechanism associated with the biocontrol potential of *Streptomyces viridodiasticus* against *Sclerotinia minor* (El-Tarabily et al., 2000). Similar observation was also reported in control of *Phytophthora fragariae* (Valois et al., 1996), *Fusarium oxysporum* (Singh et al., 1999) and other phytopathogens (Cretoiu et al., 2013).

Microbial enzymes, especially chitinases, are highly important in biocontrol strategies due to their ability (a) to degrade chitin (the main component of the cell wall of most fungi), (b) to inhibit the germination of fungal spores and the elongation of the germinative tube, and (c) to lyse hyphae (Kishore et al., 2005).

In this study, 100% of the isolates produced indole-3-

acetic acid (ranging from 6.7 to 30.7 $mg.L^{-1}$ IAA), siderophores (93.33%), phosphate solubilization (93.33%) and fix nitrogen (86.6%) (Table 1). This result was higher than that reported by Oliveira et al. (2010) who observed IAA production by 72.1% of actinobacteria tested, 86.8% solubilized phosphates and 16.2% produced siderophores. Among the 15 actinomycetes isolates tested by Jog et al. (2012), 78% produced auxins (ranging from 2.6 to 19.22 $mg.L^{-1}$ IAA equivalents), 60% produced siderophores (hydroxamate type) and only five solubilized phosphate on buffered tricalcium phosphate agar.

Although, several bacterial siderophores differ in their ability to sequester iron, as a rule these structures deprive fungi of this essential element due to their higher affinity for it (Loper and Henkels, 1999). Some bacteria which are plant growth promoting may go beyond, and attract iron from heterologous siderophores which were produced by co-inhabiting microorganisms (Lodewyckx et al., 2002). In this sense, the microorganisms selected in the present study may play a beneficial role in plant development, since growth promotion effects are also associated with the production of IAA (Khamna et al., 2009) and phosphate solubilization (Hamdali et al., 2008).

Despite its importance, phosphate solubilization has been reported in a small number of microorganisms (Hameeda et al., 2008). However, the absence of detection does not mean that the microorganism does not have this property. Rather, it may just indicate that the conditions used to detect it are not suitable. Li et al. (2015) observed that the solubilization of $Ca_3(PO_4)_2$ or MnO_2 was not detected between the *Trichoderma* inoculated cultures and the controls, but analyses of P in the calcium phytate medium revealed measurable concentrations of soluble P, significantly different from the control concentrations. It is suggested that phytase released by *Trichoderma* played a major role in solubilizing organic P (phytate) (Li et al., 2015). It was observed that increasing the incubation temperature of 25 to 30°C increased the isolates number with the ability to produce siderophores, phosphates solubilization and nitrogen fixing in 20, 28.5 and 40%, respectively. This results corroborate those found by Rinu and Pandey (2010) who observed phosphate solubilization by different *Aspergillus* species at a maximum of 28 or 21°C and the biomass production was favored at 21 or 14°C. Conversely, *A. nidulans* and *A. sydowii* exhibited maximum phosphate solubilization at 14°C and biomass production at 21°C.

In the rhizosphere soil, root exudates are the natural source of tryptophan for rhizospheric microorganisms, which may increase the biosynthesis of auxin on this site (Khamna et al., 2009). In the present study, isolates 6(4), 6(2) and 16(3) colonized the root system of seedlings of the two wheat cultivars used. This result suggests the possibility that high levels of tryptophan are present in wheat root exudates, which may enable the biosynthesis of large amounts of IAA, as the efficient colonization of roots.

Isolate 6(2) was selected for testing *in vivo* because it presented significant antifungal activity in a wide temperature range, especially at low temperatures (20°C) as well as provide a positive activity for all enzymatic activity tests, physiological and CVOs assay. The microbiolization and application of bacterial suspension to the substrate did not prevent infection of wheat seedlings by *B. sorokiniana*, as well as the extension leaf necrosis (Table 3). However, soil infestation with the actinobacteria (Treatment 4) promoted further development of wheat seedlings, with a significant increase in fresh weight, dry weight and an average number of tillers per plant for both cultivars.

Similar results were reported by Jog et al. (2012), when wheat plants were inoculated with actinomycetes isolates; they observed a high number of root branches, the number of branches, and significant biomass as compared to un-inoculated control. However, a significant change in root length was not observed by these authors. The results obtained for soil infestation with the antagonist were higher than those obtained from seeds microbiolization. This result probably was because the low concentration of bacterial inoculum adhered to the seed as compared to treatment with the soil infestation, where the inoculum concentration was much higher.

The nutrients, mostly available from plants, are broadly termed Rhizodeposition and enzymes are essential for the properly utilization of nutrients available in the rhizosphere. Many factors contribute to constructing a nutrient pool containing polymers, sugars, peptides and amino acids, organic phosphates, among others in the rhizosphere (Jog et al., 2012).

Several studies investigated the potential of secondary metabolites of actinobacteria to be used in control of diseases caused by phytopathogens and plant growth promoting. In this study, actinobacterial isolates were tested for the production of extracellular metabolites often associated with biocontrol strategies, with excellent *in vitro* results for three of these actinobacteria. These isolates were capable of utilizing nutrients such as indole-3-acetic acid, inorganic phosphates, iron, chitin, glucan and fix nitrogen, also have antifungal activity, VCOs production and rhizosphere colonization. The data shows that activities were extremely beneficial for wheat plants. Isolate 6(2) was able to promote an increased biomass and tillers per plant. Gopalakrishnan et al. (2014), in a rice field study observed that six isolates of actinomycetes significantly enhanced tiller numbers, panicle numbers, filled grain numbers and weight, stover yield, grain yield, total dry matter, root length, volume and dry weight over the un-inoculated control.

The isolates from this work may be seen as potential agents in the control of *B. sorokiniana*. However, further studies should be carried out to obtain more conclusive

results on the effectiveness of these actinobacteria against spot blotch *in vivo*.

Conflict of interest

The authors have not declared any conflict of interest

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Effect of temperature, pH and substrate composition on production of lipopeptides by *Bacillus amyloliquefaciens* 629

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The lipopeptides surfactin, fengycin and iturin produced by *Bacillus* species have diverse ecological roles, including antimicrobial activity, induced systemic resistance in plants against pathogens and plant colonization by the producing strain. The conditions that govern both lipopeptide production and plant colonization are not fully understood. The present study investigated the role of growth media, temperature and pH on the production of the lipopeptides surfactin, fengycin and iturin by *Bacillus amyloliquefaciens* 629 and its production in bean plants colonized epiphytically and endophytically by this isolate. Surfactin was produced at higher amounts when isolate 629 was grown at 15 than at 25 and 30ºC, whereas fengycin remained approximately constant across different temperatures. Iturin was detected on bean stem sap, root exudates and in potato dextrose broth, indicating that plant-derived nutrients play an important role in its production by isolate 629. *B. amyloliquefaciens* 629 colonized plants with more efficacy at 28 than at 20°C. None of the lipopeptides was detected in plants colonized by isolate 629, despite the number of attempts performed with ultra-performance liquid chromatography (UPLC) analysis.

Key words: Bioaccumulation, fengycin, iturin, surfactin.

INTRODUCTION

Lipopeptides are surface-active molecules that may possess antimicrobial activity, induce plant immune responses and may facilitate plant colonization. These compounds play a role in the ecological fitness of the producing organism (Yoshida et al., 2001; Hsieh et al., 2008; Richardson et al., 2009; Raaijmakers et al., 2010;

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Cawoy et al., 2015). Among many active compounds produced by *Bacillus* spp., surfactins, fengycins and iturins are the most frequently reported lipopeptides with a role in biological control of phytopathogens. Surfactins, fengycins and iturins are families of compounds containing chemical variants of each of these lipopeptides (Ongena and Jacques, 2008). Surfactins are bioemulsifiers that promote increased surface area for hydrophobic water-insoluble growth substrates; bioavailability of hydrophobic substrates by increasing solubility; and attachment and detachment of bacteria to and from surfaces (Rosenberg and Ron, 1999). Fengycin and iturins have mostly an antimicrobial role, mainly in the displacement of filamentous fungi and some groups of bacteria from substrates (Vanittanakom et al., 1986, Steller et al., 1999). Lipopeptides play an important role in the modes of action employed by *Bacillus* in the protection of plants against diseases (Raaijmakers et al., 2010). Synergistic effects of surfactins, fengycins and iturins in the control of phytopathogens were reported earlier by Maget-Dana et al. (1992), Ongena et al. (2007) and Romero et al. (2007).

Production of lipopeptides is regulated by a two-component system in a quorum-sensing dependent manner (Duitman et al., 2007). Surfactins are synthesized during the transition from the exponential to the stationary bacterial growth phase, whereas the biosynthesis of fengycins and iturins occurs later in the stationary phase (Vater et al., 2002). Some abiotic factors were shown to interfere with lipopeptide production by *B. subtilis* (Mizumoto and Shoda, 2007; Vater et al., 2002).

Plants interact with microorganisms that surround their tissues and the root system has the strongest influence on this interaction. Plant roots release sugars, amino acids, organic acids and inorganic ions that support the growth and production of bioactive molecules. For instance, *B. amyloliquefaciens* strain S499 produces surfactin, fengycin and iturin in tomato root exudates (Nihorimbere et al., 2012).

Once colonized by beneficial bacteria, plant roots experience long-term benefits, such as increased growth, increased resistance to chemical and physical damage, and direct antagonism against harmful plant pathogens (Punja, 2001; Yoshida et al., 2001; Richardson et al., 2009). Colonization is the first step and a prerequisite for the successful delivery of these benefits to host plants (Steenhoudt and Vanderleyden, 2000). Little is known, however, on the influence of lipopeptides on plant colonization.

Since lipopeptides play a crucial role in the ecological fitness of *Bacillus* species, the objectives of this work were to study: 1) the lipopeptide production in different substrates (MOLP, LURIA, PDB and MB1) and temperatures (30, 25 and 15°C) by *B. amyloliquefaciens* 629; 2) the influence of the substrate pH in lipopeptide production; 3) lipopeptide production using stem sap and root exudates as a sole source of nutrients and growth factors; 4) the endophytic and epiphytic bean plant colonization at 28 and 20°C and the quantification of lipopeptides *in planta*.

MATERIALS AND METHODS

Bacterial isolate and growth conditions

Bacillus isolate 629 was isolated from a healthy adult *Theobroma cacao* tree (Leite et al., 2013). Its identity was confirmed as *B. amyloliquefaciens* on the basis of 16S rDNA (JQ435867), *gyr*A (LN555733) and *rec*A sequences (LN555734). The isolate was deposited in the Biological Institute Culture Collection of Phytopathogenic Bacteria - IBSBF (Campinas, São Paulo, Brazil) under accession number IBSBF-3106. This collection was registered with the World Data Centre for Microorganisms collection under number WDCM-110. A spontaneous rifampicin-resistant (rif^R) variant of 629 able to grow on 100 µg/mL of this antibiotic was used in all experiments. Isolate 629 was grown on liquid MOLP, MB1, Luria-Bertani Broth and PDB at 15, 25 and 30°C for 48 h. The media composition were as follows: MOLP medium (casein peptone 30 g/L, saccharose 20 g/L, yeast extract 7 g/L, KH_2PO_4 1.9 g/L, $MgSO_4$ 0.45 g/L, citric acid 10 mg/L, $CuSO_4$ 0.001 mg, $FeCl_3.6H_2O$ 0.005 mg, $NaMoO_4$ 0.004 mg, KCl 0.002 mg, $MnSO_4.H_2O$ 3.6 mg, $ZnSO_4.7H_2O$ 0.014 mg and H_3BO_3 0.01 mg; pH was adjusted to 7 with KOH) (Ahimou et al., 2000); MB1 liquid (sucrose 10 g/L, casein peptone 8 g/L, yeast extract 4 g/L, K_2HPO_4 2 g/L, $MgSO_4.7H_2O$ 0.3 g/L) (Kado and Heskett, 1970); modified Luria-Bertani Broth (casein peptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L and glucose 1 g/L) (Miller, 1972); and PDB (potato dextrose broth 24 g - Difco). Bacterial suspensions were prepared by growing isolate 629 for 24 h in liquid MB1 medium at 30°C under 120 rpm and adjusted to concentrations that depended on the experiment. The bacterial isolate was stored in 40% glycerol at -80°C.

Influence of culture media, temperature and pH on lipopeptide production

Lipopeptide concentrations were studied in the four different culture media described above and at three temperatures. Erlenmeyer flasks (125 mL holding capacity) containing 25 mL of each culture medium received 50 µL of a *B. amyloliquefaciens* 629 suspension at $2x10^5$ CFU/mL. The treatments with 3 replicates were placed in an orbital shaker at 120 rpm and 15, 25 and 30°C and incubated for 72 h. In PDB medium at 15°C, the bacterium was incubated at up to five days. All experiments were done twice.

To study the production of lipopeptides in different pHs, the MB1 was arbitrarily chosen and 100 µL of a suspension of isolate 629 containing $2x10^5$ CFU/mL was transferred to Erlenmeyer flasks containing 50 mL of medium adjusted to pH 5, 6, 7, 8 and 9. The flasks were incubated in an orbital shaker at 120 rpm at 30°C for 72 h and at the end of the experiment the final pH was measured. Experiments were installed in a completely randomized design with 3 replicates and were done twice.

Extraction and detection of lipopeptides produced *in vitro* was performed by collecting samples of 12 mL from the experiments to analyze medium composition and pH, centrifuged for 10 min at 25,155 g and 10 mL of supernatant was passed through C18 solid-phase extraction cartridges (Grace Maxi-CleanTM SPE 300mg, Alltech Associates Inc., Lokeren, Belgium), previously rinsed with 20 mL methanol and 15 mL MilliQ water, followed by a wash with 5 mL MilliQ water. After this, the lipopeptides adhering to the column were released by passing 1 mL of methanol through the cartridge and transferred to 1.5 mL microcentrifuge tubes. The samples were centrifuged again for 10 min at 25,155 *g* and 300 µL were transferred

to a special tube for ultra-performance liquid chromatography - UPLC analysis (Waters Acquity H-class). The UPLC was coupled to a single mass spectrometer (Waters Single Quad Detector - SQD mass analyzer). UPLC analysis was carried out on a reverse phase column (Acquity UPLC BEH C18 1.7 µm, 2.1 X 50 mm, Waters). Elution was performed at 40°C with a constant flow rate of 0.6 mL/min using a gradient of acetonitrile in water acidified with formic acid (0.1%) as follows: 30% acetonitrile for 2.43 min, from 30 to 95% for 5.2 min, then the column was stabilized at 30% acetonitrile for 1.7 min. The lipopeptides were detected in electrospray positive ion mode by setting the SQD parameters as follows: source temperature, 130°C; desolvation temperature, 400°C; and nitrogen flow, 1000 $L.h^{-1}$. A cone voltage of 120 V was used (Cawoy et al., 2015). After the analyses, the lipopeptides were identified in the chromatogram and spectrogram by mass comparison and retention time.

Production of lipopeptides in bean stem sap and root exudates

Common bean seeds (*Phaseolus vulgaris* - cultivar Pérola) were sown in vermiculite and incubated for 10 days in a chamber adjusted to 28°C. The stems were separated from the leaves and roots, surface-disinfested with alcohol (70% v/v) for 30 s, sodium hypochlorite (2% v/v) for 1 min, and washed three times with sterile distilled water. Forty stems (7.83 g) were transferred to a 1-L beaker with 600 mL of sterile distillated water and incubated at 28°C for 48 h under sterile conditions to extract the stem sap, which was later passed through a 0.22 µm diameter filter to eliminate stem residues. To obtain root exudates, roots of 40 plants (37.56 g) were surface-sterilized, incubated, and filtered as described for the stem sap. Aliquots of 20 mL of the stem or root exudates were transferred to Erlenmeyer flasks (125 mL), and 100 µL of the bacterial suspension ($2x10^5$ CFU/mL) was added to each of the three flasks that constituted the replicates of the experiment that was performed twice in a completely random design. The samples were incubated in an orbital shaker at 120 rpm and 30°C for 120 h, then lipopeptides were analyzed as described above.

Plant colonization and lipopeptide production

These experiments were performed in sterile glass bottles filled with vermiculite and Hoagland's solution was used as the nutrient and water source (Hoagland and Arnon, 1950). Bean seeds were pre-germinated in the vermiculite for 5 days and inoculated by dipping the roots for 30 min in a suspension containing $2x10^5$ CFU/mL. Subsequently, the inoculated plants were incubated at 28°C or 20°C with a 12 h photoperiod for 10 days. Endophytic and epiphytic populations of isolate 629 in the same plant were assessed separately on solid MB1 supplemented with rifampicin. To assess endophytic population, roots, stems and leaves were separated, weighted, surface-sterilized, ground in a mortar and pestle and 10x step dilutions prepared in 0.5% saline solution were plated on MB1 and incubated for 24 h at 30°C. Only samples that showed no bacterial growth after plating aliquots from the third rinse with distilled water during the surface sterilization were used for estimating bacterial population. This procedure was adopted to confirm the endophytic nature of the bacteria from these plant parts. To estimate the epiphytic populations, each plant part was immersed in 0.5% saline solution, vortexed for 1.5 min and 10x step dilutions were plated on MB1 as described above. Extraction and detection of lipopeptides produced *in vivo* was performed by collecting 10-day old bean plants, separating roots, stems and leaves and grinding in a mortar and pestle with liquid nitrogen. Samples of 1 g of the resulting powder were mixed with 10 mL of extracting solution, composed of acetonitrile and 1% formic acid. Samples were dried in a SpeedVac and resuspended in 1 mL of extracting solution. Analysis of the lipopeptides was performed in an UPLC, as described above. The experiments were installed in a completely randomized design with three replicates and performed twice.

Statistical analysis

All statistical analyses were performed with the R software (R Core Team, 2014). Comparisons between the means were performed using Tukey or the t-test for paired data at 5% probability.

RESULTS

Among the different culture media tested, only PDB allowed production of all three lipopeptides at 30 and 25°C, and it was the only medium where iturin was produced, however, there was no bacterial growth at 15°C. In PDB, the production of fengycin and surfactin was the lowest as compared to the production in the other media. Production of surfactin increased as the temperature decreased for all media, except for PDB, whereas fengycin remained at an approximately constant level as the temperatures changed, but with a higher production at 25°C. In addition, the effect of the temperature was most pronounced for surfactin that had its production increased by lower temperatures (Table 1). A representative chromatogram and spectrogram shows the production of the three families of the lipopeptides detected for strain 629 (Figure 1).

The final pH of the medium adjusted to pH 5 to 7 remained similar and for the ones with initial pH of 8 and 9 were lowered to values around 7. Surfactin and fengycin were only detected at initial pH 6 and 7, with the maximum production at pH 6 and iturin was not produced at the same pH (Table 2). Bacterial population sizes did not seem to have influenced the production of lipopeptides and the variation between the lowest and the highest densities was 6.5x at the different pH levels. Stem sap allowed the production of higher amounts of lipopeptides than root exudates. Root exudates showed no production of fengycin and lower populations of bacteria than stem sap (Table 3).

Isolate 629 colonized bean plants epiphytically and endophytically and was recovered from all plant parts, including leaves, stems, and roots when plants were grown at 28°C, whereas no bacteria were recovered from leaves at 20°C. Population densities were similar among all treatments, except for leaves at 20°C (Figure 2). The lipopeptides fengycin, iturin and surfactin were not found in bean plants, irrespective of the incubation temperature and part of the plant analyzed.

DISCUSSION

B. amyloliquefaciens 629 has been reported to promote

Table 1. Detection of lipopeptide production by *B. amyloliquefasciens* 629 grown in different liquid media and incubated at three different temperatures with 120 rpm of shaking for 72 h. Lipopeptides were determined by ultra-performance liquid chromatography (UPLC) analysis.

Culture media	Temperature (°C)	Population (CFU/mL)	µg lipopeptide/mL		
			Surfactin	Fengycin	Iturin
MOPL	30	$1.5 \times 10^7 \pm 6.47 \times 10^6$**	2.92 ± 0.30^{cd}	0.82 ± 0.04^{bcd}	nd*
	25	$8.9 \times 10^6 \pm 5.64 \times 10^6$	4.51 ± 0.97^{c}	1.44 ± 0.73^{abcd}	nd
	15	$3.1 \times 10^7 \pm 2.38 \times 10^6$	9.45 ± 0.81^{b}	0.29 ± 0.10^{d}	nd
MB1	30	$9.1 \times 10^6 \pm 1.29 \times 10^6$	0.37 ± 0.03^{e}	0.44 ± 0.09^{cd}	nd
	25	$7.8 \times 10^6 \pm 1.92 \times 10^6$	5.21 ± 0.07^{c}	1.96 ± 0.04^{ab}	nd
	15	$1.2 \times 10^7 \pm 2.66 \times 10^6$	14.39 ± 2.32^{a}	1.86 ± 0.51^{abc}	nd
PDB	30	$4.9 \times 10^6 \pm 1.14 \times 10^6$	0.30 ± 0.01^{e}	0.14 ± 0.05^{d}	0.72 ± 0.14^{a}
	25	$1.1 \times 10^7 \pm 1.95 \times 10^6$	0.44 ± 0.03^{e}	0.24 ± 0.07^{d}	3.05 ± 0.25^{b}
	15	nd	nd	nd	nd
Luria	30	$6.8 \times 10^6 \pm 3.72 \times 10^6$	1.91 ± 0.88^{de}	2.04 ± 1.93^{ab}	nd
	25	$3.5 \times 10^6 \pm 1.90 \times 10^6$	3.76 ± 0.79^{cd}	2.63 ± 0.87^{a}	nd
	15	$7.1 \times 10^6 \pm 2.82 \times 10^6$	10.36 ± 2.46^{b}	0.84 ± 0.70^{bcd}	nd

Means followed by the same letter in the columns are not significantly different according to Tukey's test ($p<0.05$). *nd - not detected. **Standard deviation.

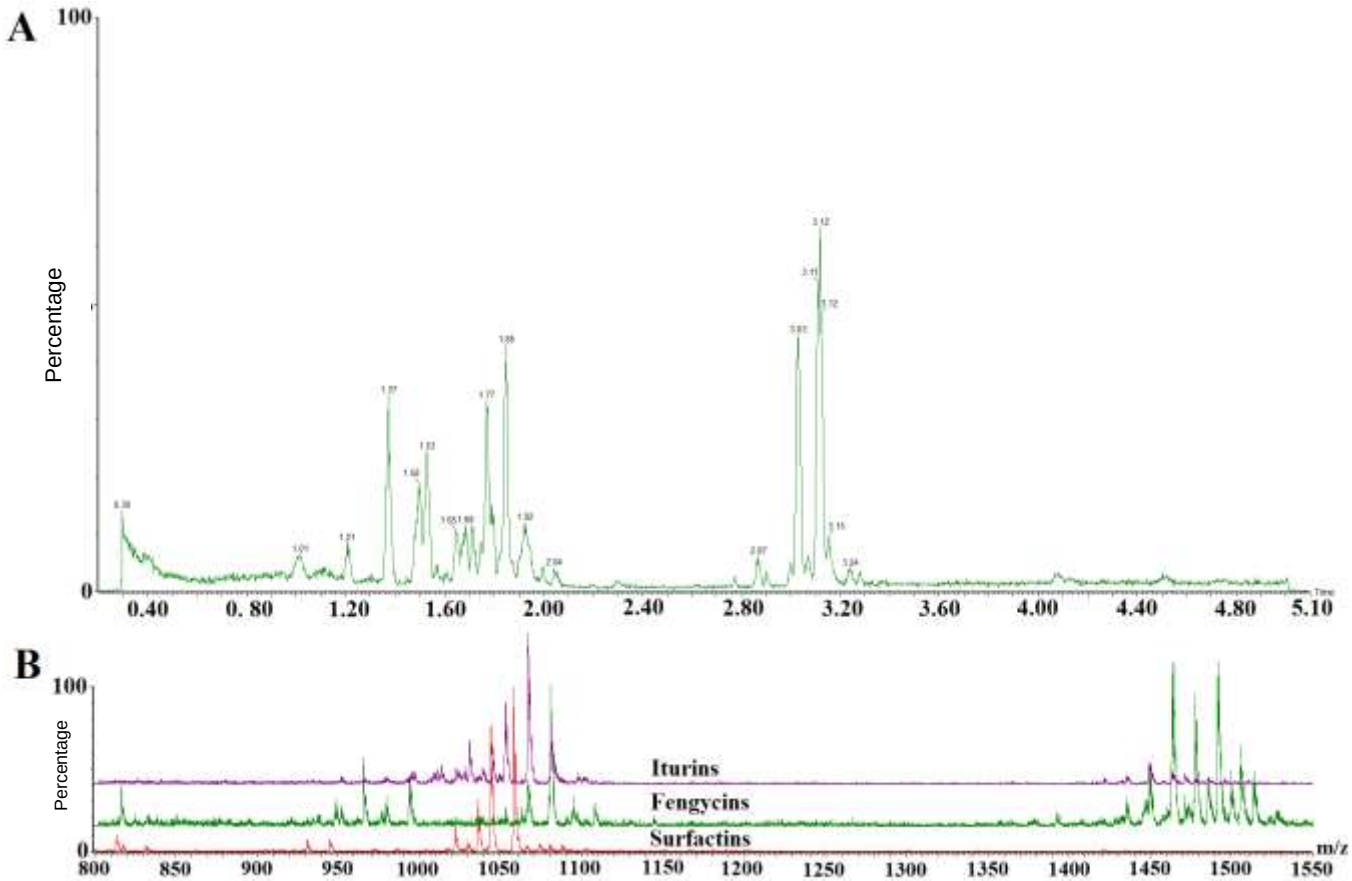

Figure 1. Lipopeptides produced by the strain 629. A representative chromatogram (A) and spectrogram (B) showing iturins, fengycins and surfactins produced by *B. amyloliquefaciens* 629 in PDB at 25°C during 72 h of incubation.

Table 2. Detection of lipopeptide production by *B. amyloliquefasciens* 629 grown in liquid MB1 medium adjusted to different initial pHs and incubated at 30°C with 100 rpm of shaking for 72 h. Lipopeptides were determined by ultra-performance liquid chromatography (UPLC) analysis. Iturin was not detected.

Initial pH	Lipopeptide	µg/ml	Final population (CFU/mL)	Final pH
5	Surfactin	nd*	$1.3 \times 10^5 \pm 1.33 \times 10^3$	5.18±0.18
	Fengycin	nd		
6	Surfactin	4.54±1.81[a]**	$3.8 \times 10^4 \pm 2.21 \times 10^2$	6.28±0.11
	Fengycin	1.72±0.14[a]		
7	Surfactin	0.53±0.02[b]	$2.0 \times 10^4 \pm 1.49 \times 10^3$	6.88±0.07
	Fengycin	0.10±0.01[b]		
8	Surfactin	nd	$5.4 \times 10^4 \pm 4.25 \times 10^4$	6.84±0.17
	Fengycin	nd		
9	Surfactin	nd	$6.3 \times 10^4 \pm 4.46 \times 10^4$	6.88±0.27
	Fengycin	nd		

Means with the same letter are not significantly different according to the t-test ($p<0.05$). Only means of the same lipopeptide in different pH were compared. *nd- not detected. **Standard deviation.

Table 3. Detection of lipopeptide production by *B. amyloliquefasciens* 629 grown in bean stem sap and root exudates and incubated at 30°C with 120 rpm of shaking for 120 h. Lipopeptides were determined by ultra-performance liquid chromatography (UPLC) analysis.

Plant organ	Population (CFU/mL)	Lipopeptides (µg/mL)		
		Surfactin	Fengycin	Iturin
Stem sap	$1.8 \times 10^4 \pm 1.65 \times 10^2$**	0.46±0.21[a]	0.03±0.02	2.23±1.05[a]
Root exudate	$6.7 \times 10^3 \pm 4.03 \times 10^3$	0.03±0.01[b]	nd*	0.08±0.06[b]

Means with the same letter are not significantly different according to the t-test ($p<0.05$). Only means of the same lipopeptide were compared. *nd - not detected. **Standard deviation.

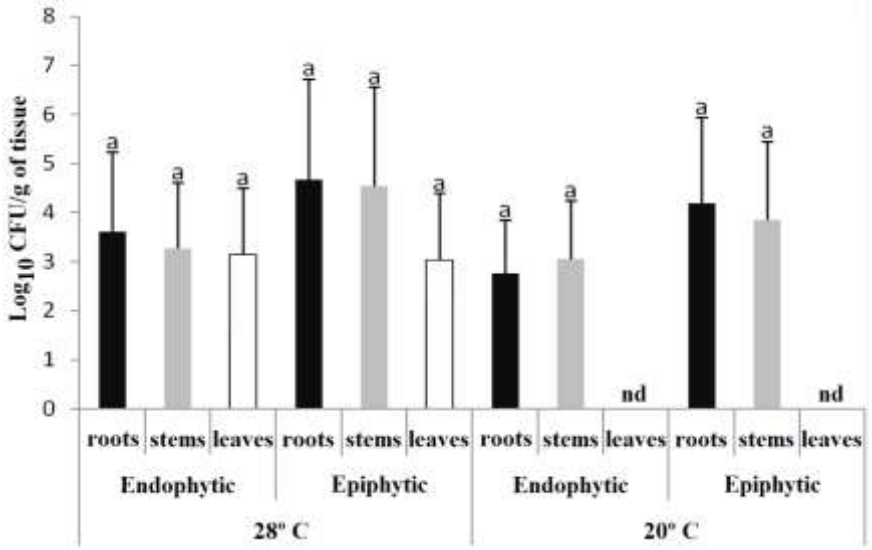

Figure 2. Endophytic and epiphytic colonization of bean plants by *B. amyloliquefaciens* 629. Inoculated plants were cultivated inside glass bottles with vermiculite and incubated at 28 and 20°C for 10 days. Bacterial populations were determined in (endophytic) and on (epiphytic) different plant parts by plating dilutions on solid MB1 medium with rifampicin. Error bars represent the standard error of the means. Means followed by the same letter are not significantly different according to Tukey's test ($p<0.05$). nd- not detected.

cacao growth and to have an antagonistic effect against witches' broom etiologic agent, *Moniliophthora perniciosa* (Falcao et al., 2014), and to reduce the bacterial wilt severity caused by *Curtobacterium flaccumfaciens* pv. *flaccumfacies* (Martins et al., 2013). In addition, strain 629 increases magnesium content in the common bean shoots (Martins et al., 2015).

The lipopeptides fengycin, surfactin and iturin are commonly produced by *Bacillus* species in common culture media (Akpa et al., 2001; Mukherjee and Das, 2005). In this study, it was found that *B. amyloliquefasciens* 629 produces the lipopeptides iturin, fengycin and surfactin in a temperature- and medium composition-dependent manner. In the first step of the experiments, isolate 629 only produced iturin in PDB; although, all tested culture media supported similar bacterial densities. The optimized medium referred to as MOLP (Ahimou et al., 2000) was not the best substrate for lipopeptides production by strain Alb 629.

Surfactin acts as a surfactant and lowers the surface tension to facilitate swarming motility and also was shown to induce systemic resistance (Sachdev and Cameotra, 2013; Cawoy et al., 2014; Phae and Shoda, 1990; Hsieh et al., 2008). Fengycin is also an antimicrobial lipopeptide, but does not have the broad-spectrum activity of iturin (Ongena et al., 2010).

The initial pH close to neutral or slightly acidic favored the production of surfactin and fengycin, coinciding with the optimal pH range for bacterial growth, as demonstrated by Makovitzki and Shai (2005) and Mandal et al. (2013).

Lipopeptides including iturins were also produced in bean stem sap and in root exudates, but they were not detected in bean plants colonized epiphytically and endophytically by isolate 629, even after several attempts. Tissue specific induction of lipopeptide production is likely to occur, since stem sap exudates induced a 15- and 27-fold increase in the concentrations of surfactin and iturin, respectively, while it only promoted a 2-fold increase in bacterial populations. This result suggests that plant-derived factors contribute to the regulation of iturin production by isolate 629, as also observed by Raaijmakers et al. (2010).

Although, surfactin plays a significant role in the colonization of tomato root surfaces (Nihorimbere et al., 2009), it does not seem to be important in the acropetal movement of isolate 629 in bean plants, since isolate 629 was not detected in leaves at 20°C, which is a favorable temperature for the production of the lipopeptide *in vitro*.

It is postulated that either the adopted methods are not suitable for lipopeptide detection *in vivo* or the plant has the ability to interact or modify the molecules, turning them into another compound (Suga and Hirata, 1990). Other plants may induce a differential production of lipopeptides and may be tested in future studies allied with the use of more sensitive techniques such as time of flight – secondary ion mass spectrometry (TOF-SIMS) (Nihorimbere et al., 2012).

Conclusions

Lipopeptides production is both qualitatively and quantitatively affected by differences in substrate, temperature and pH. Surfactin is the most influenced by the temperature, while pH range of 6 and 7 favor the production of fengycin and surfactin. Iturin is only produced in media containing plant-derived nutrients. Although, *B. amyloliquefaciens* 629 colonizes plants endophytically and epiphytically, none of the lipopeptides were detected in bean plants.

Conflict of Interests

The authors have not declared any conflict of interests.

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Isolation, screening and statistical optimizing of L-methioninase production by *Chaetomium globosum*

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Egyptian soil sample was screened for isolation methioninlytic fungi by a rapid plate assay procedure. Eighteen strains of different isolated fungi were screened quantitavely for their L-methioninase activity. *Chaetomium globosum* was the most efficacious isolate and a dematiaceous filamentous fungi, it was identified at the molecular level by ribotyping 18S rRNA along with the biochemical characterization, and lastly completed by BLAST analysis by structure of a phylogenetic tree. Results showed that, the optimum levels of the incubation period, temperature, pH, methionine, sucrose and sodium nitrate concentrations were 3 days, 30°C, 7, 0%, 30 g/l and 1g/l, respectively. According to the produced model, at these levels, *C. globosum* produce L-methioninase with predicted specific activity of (≈2225 U/mg); so L-methioninase could be a good source for clinical therapeutic application. The rRNA sequence of *C. globosum* was deposited to gene bank under accession number KXO24450

Key words: L-methioninase, *Chaetomium globosum*, 18S rRNA, enzyme optimization, soil.

INTRODUCTION

L-Methioninase (E.C 4.4.1.11) is a pyridoxal phosphate-dependent enzyme and is a fulfilling several functions enzyme system because it stimulates the, γ- and α, β-removal reactions of methionine and its derivatives. Physiologically, normal cells have the capability to grow on homocysteine, instate of methionine, due to their efficient methionine synthase (Mecham et al., 1983). Unlike normal cells, tumor cells freed from efficient methionine synthase thus rely on external methionine supplementation from the diet (Hoffman, 1984). So, L-methioninase has extradited reasonable heed as a therapeutic agent against different kind of methionine dependent tumors (Weisendanger and Nisman, 1953; Kokkinakis et al., 1997). Methionine reduction has a broad spectrum of antitumor activities (Kokkinakis, 2006). Under methionine reduction, tumor cells were blocked in the late S-G2 phase because the pleiotropic influences and suffered apoptosis. Thus, therapeutic making use of L-Methionine γ-lyase to reduction plasma methionine has been widely examined (Yoshioka et al., 1998; Hoffman., 2015). L-Methioninase is present in most of organisms, as bacteria, fungi, protozoa, and plants, except mammals. L-Methioninase was at first described from the rumen bacteria (Weisendanger and Nisman, 1953; Miwatani et al., 1954). A few studies were concentrated on the enzyme from eukaryotes (especially fungi), comparing it to the bacterial sources, even though the therapeutic response of the bacterial enzyme is commonly related

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with high immunogenicity, low substrate specificity, and risky influences to the kidney and liver (Sun et al., 2003). Like many bacterial and fungal species, the enzyme was detected in the cell-free extract (Lockwood and Coombs, 1991; Tokoro et al., 2003). L-methioninase was detected from different species of bacterial such as intracellular enzyme (Tanaka et al., 1976), the intracellular nature of bacterial L-methioninlytic enzyme may have extra limiting step during the scale production and also from fungi as intracellular and extracellular enzyme (Ruiz-Herrera and Starkey, 1969; El-Sayed, 2009). Due to the recurrent classification of L-methioninase as extracellular enzyme in the fungal extract, fungi could be considered as robust resources to this enzyme. As it was notified for other fungal enzymes, the extracellular output is more the intracellular one by four fold (Pandey et al., 1999; El-Sayed., 2008). *C. globosum* Kunze is a saprophytic fungus which is member of a genus whose species are described as cellulytic organism (Lakshmikant and Mathur., 1990). Due to their bio-deterioration ability, several strains are used in testing materials for mould growth resistance. *C. globosum* genes encoding putative proteins lead to the identification of 30 genes, 27 of which includes the degradation of various cell wall polymers (Longoni et al., 2012). According to the former researches, the production and purification of L-methioninase were fundamentally proceeds under submerged conditions (Bonnarme et al., 2000; Amarita et al., 2004; Martinez-Cuesta et al., 2006). L-methionine as many amino acids can be formed Amadori compounds through Millard reactions (Delgado-Andrade et al., 2007) that decrease their bioavailability as carbon and nitrogen for the organism. Screening for a new producers and new kinds of the growth medium for the bulk production of this enzyme with additional therapeutic characters to fulfill their requirements will be a challenge. Our work concentrated on screening for L-methioninase-producing fungi isolated from Egyptian soil and using statistical (response surface) methodology in a trial to reduce or eliminate L-methionine from the formulated medium in parallel with optimization of the production conditions.

MATERIALS AND METHODS

L-Methionine, sodium nitrate, sucrose, sodium nitroprusside, Nessler and Bradford reagent were of analytical grade.

Isolation and purification of methioninolytic fungi

The dilution-plate method was used for isolation of Egyptian soil fungi capable L-methioninase production as described by Johnson et al. (1959). Using modified medium, contains methionine (5 g/l), glucose (20 g/l), $NaNO_3$ (2 g/l), KCl (0.5 g/l), K_2HPO_4 (1 g/l), $MgSO_4$. $7H_2O$ (0.5 g/l), all dissolved in 1 L of distilled water. The final pH of the medium was adjusted to 6.0. The final plates were incubated at 28°C for 7 days, and the developed fungal isolates were purified on the same medium.

Screening for L-methioninase producer strains

The fungal isolates were screened for their L-methioninase productivities using qualitative rapid plate assay using the above medium and phenol red was added to the medium as indicator at final concentration of 0.007% just before pouring the plate and it incubated at 28°C for 7 days (Sundar and Nellaiah, 2013).

Morphological identification of methioninolytic fungi

Identification of the isolated fungi during our investigation was carried out using the morphological characteristics as colony diameter, the color of condia, extracellular exudates, pigmentation and the color of reverse mycelium and microscopic features were examined also as conidial heads, fruiting bodies, degree of sporulation and the homogeneity characters of conidiogenous cells by optical light microscope (10×90) Olympus CH40 according to the following studies: Barron (1968), Booth (1971, 1977), Ainsworth (1971), Ellis (1971, 1976) and Pitt (1985). Fungal isolates were grown onto malt extract-agar (MA) medium at 28°C for several days (7-10). The cultures were then kept in 4°C.

Molecular identification of methioninase producer fungi

Preparation of the fungal culture

The spores of 4 days old culture of tested fungi were collected by addition of sterile saline (5 ml) to slant and the suspension was inoculated to 100 ml of Czapek Dox's medium in 250 ml Erlenmeyer flask. After incubation for 4 days, the cultures were filtered and the mats were collected and washed with distilled water.

Genomic DNA extraction

Genomic DNA of fungi was extracted according to Sharma et al. (2007) as the following; 50 to 100 mg fungal mycelia were homogenized. Five hundred microliter of DNA extraction buffer (200 mM Tris-HCl pH 8, 240 mM NaCl; 25 mM EDTA, and 1% SDS) were then added to the homogenized fungal materials. One volume of phenol/$CHCl_3$, in the ratio of 1:1 (v/v), was added and mixed gently for 10 min on a shaker followed by centrifugation at 15000 x g for 10 min. The upper phase was transferred to a new tube and 0.1 vol of 3 M Na-acetate (pH 5.2) and 2 volume of ethanol (96%) were added and mixed well, incubated for 30 min at -20°C followed by centrifugation (15000 x g/4°C/20 min). The resulting pellet was washed with 700 µl of 70% ethanol, air dried and re-suspended in 100 µl of sterile bi-distilled water.

PCR amplification

The primers ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3' were used for the PCR. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30 µl reaction mixture by using a *EF-Taq* (Sol Gent, Korea) as follows: Activation of Taq polymerase at 95°C for 2 min, 35 cycles of 95°C for 1 min, 55°C, and 72°C for 1 minutes each were performed, finishing with a 10- minute step at 72°C. The

Table 1. Experimental design matrix prepared using Taguchi orthogonal array (OA) for L-methioninase production.

Run order	Incubation time (days)	Temperature (ºC)	pH	Methionine Conc. (g/l)	Sucrose (g/l)	Sodium nitrate (g/l)
1	7	35	4	5	30	1.5
2	3	40	8	10	30	3
3	3	20	4	0	10	1
4	11	30	5	0	30	2.5
5	5	20	5	5	25	3
6	3	25	5	2.5	15	1.5
7	7	30	8	2.5	25	1
8	7	25	7	0	20	3
9	5	40	4	2.5	20	2.5
10	11	35	6	2.5	10	3
11	9	40	6	0	25	1.5
12	9	30	4	7.5	15	3
13	9	20	7	2.5	30	2
14	3	35	7	7.5	25	2.5
15	11	40	7	5	15	1
16	11	25	4	10	25	2
17	7	20	6	10	15	2.5
18	9	35	5	10	20	1
19	11	20	8	7.5	20	1.5
20	7	40	5	7.5	10	2
21	5	35	8	0	15	2
22	9	25	8	5	10	2.5
23	3	30	6	5	20	2
24	5	25	6	7.5	30	1
25	5	30	7	10	10	1.5

amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM Big Dye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95°C for 5 min, followed by 5 min on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

Production of L- methioninase

The higher producing isolates for L-methioninase were taken up for studies on enzymes production in the same medium. The conidial suspension was prepared by injecting 10 ml of sterilized saline solution (0.85%) into a 7 day old slant of selected fungus. 1 ml of spore suspension was inoculated in 30 ml of modified liquid Czapek-Dox medium containing methionine in 100-ml Erlenmeyer conical flasks. The submerged cultures were incubated at 28°C for 7 days at 120 rpm.

Experimental design

Response surface method; Taguchi orthogonal array (OA) model (Table 1) based on six factors, five levels each, was used to study the effect and interactions between different factors; incubation time (days), temperature (ºC), pH, methionine concentration (%), sucrose concentration (%) and sodium nitrate concentration (%) for maximum production of L-methioninase enzyme (U/mg protein). Experimental designs were performed using Design-Expert software (Stat-Ease Inc., Minneapolis, MN, USA, ver 7.0.0). Experimental significance of the obtained model was checked by F-test (calculated p-value) and goodness of fit by multiple correlation R as well as determination R^2 coefficients. L-Methioninase specific activity (U/mg protein) was measured as an experimental response.

Statistical analysis

Analysis of variance (ANOVA) was used to estimate the statistical parameters for maximum productivity of L-methioninase. A probability value of p value <0.05 was used as the criterion for statistical significance.

Methioninase assay

L-Methioninase activity was assayed by direct Nesslerization according to the method of Thompson and Morrison (1951) with some modifications. The standard reaction system contains 1 ml of 1% L-methionine in phosphate buffer (pH 7.0), 0.1 ml of pyridoxal

Table 2. Screening for L-methioninase production by different fungal strains.

Scientific name	L-methioninase activity
Penicillium sp. Pitt	-ve
Penicillium janthinellium Biourge	-ve
Aspergillus niger Van Tieghem	-ve
Aspergillus joponicus Saito	-ve
Aspergillus aculeatus Iizuka	-ve
Aspergillus sp. link	-ve
Penicillium nigricans (Bainier) Thom	-ve
Fusarium sp. Link	-ve
Collectrichum sp. (Sacc. & Magnus) Briosi & Cavara.	++
Penicillium digitatum (Persoon Ex Fr.) Saccardo	-ve
Pythium sp. Pringsh.	++
Fusarium oxysporium Snyder & Hansen	+
Penicillium glabrum (Wehmer) Westling	-ve
Aspergillus ochraceus Wihelm	-ve
Fusarium poae (Peck) Wollenw	-ve
Chaetomium globosum Kunzen, Fries.	+++
Aspergillus flavus Link	-ve
Aspergillus cervinus Massee	++

phosphate, and 1 ml of crude enzyme. The reaction system was incubated at 30°C for 1 h. The enzymatic activity was stopped by adding 0.5 ml of 1.5 mol/l trichloroacetic acid or by boiling for 5 min. The system was centrifuged at 5,000 rpm for 5 min to remove the precipitated protein. 0.1 ml of above mixture was added to 3.7 ml of distilled water and the released ammonia was determined using 0.2 ml of Nessler reagent, and the developed colored compound was measured at 480 nm using UV/VIS-2401 PC visible spectrophotometer (Shimadzu, Kyoto, Japan). Enzyme and substrate blanks were used as controls. One unit of L-methioninase was defined as the amount of enzyme that liberates ammonia at 1 µmol/h under optimal assay conditions. The specific activity of L-methioninase was expressed as the activity of enzyme in terms of units per milligram of protein.

Determination of extracellular protein

The protein concentration of the prepared crude was estimated by Bradford reagent according to Bradford (1976).

Determination of methionine uptake

The residual methionine of culture filtrate was determined on the basis of the thioether group according to the method of Hess and Sullivan (1943) with some modifications. Using this method, 1 ml of the supernatant was mixed with 0.5 ml of 3% glycine, 1 ml of 2% sodium nitroprusside, and 0.5 ml of 1 N NaOH. The mixture was incubated in a water bath at 40°C for 15 min., then chilled in an ice bath for 5 min. Next, 1 ml of a 1:9 (v/v) mixture of H_2SO_4:H_3PO_4 was added with vigorous shaking for 5 min. The developed color was measured spectrophotometrically at 530 nm. The methionine concentration was determined from the standard curve of methionine prepared under the same conditions. The rate of methionine uptake was expressed as the amount of consumed methionine/initial methionine concentration $\times$100.

Biomass determination

After the fermentation process, the cultures were centrifuged at 5,000 rpm for 10 min at 4°C followed by filtration through Whatman no. 1 filter paper. The cell pellets were washed with distilled water and dried at 80°C until a constant weight was achieved. The dry biomass was expressed as grams per liter of fermentation medium.

RESULTS AND DISCUSSION

Screening for L-methioninase production by soil fungi

The screening profile (Table 2) shows the ability of different fungal strains to produce L-methioninase

From 18 terrestrial fungi screened, only five isolates were identified as methioninlytic fungi as manifested by the pink color of the colonies, generating from the manufacture of ammonia by the action of L-methioninase on L-methionine (Table 2). Production of L-methioninase by *Fusarium oxysporium* was previously recorded (Bahl et al., 2012) but from endophytic isolate not terrestrial isolate like in our study. *Aspergillus* species are used in commercial enzyme production, more than 80 recombinant enzymes of fungal origins are universally used, and 55 of these proteins were produced from *Aspergillus* species (Yoder and Lehmbeck, 2004). According to our data, it is first record for production L-methioninase by *Aspergillus cervinus* and also it is first

Table 3. L-Methioninase productivity, methionine uptake and dry weight by *C. globosum* based on actual, predicted and residual values according to Taguchi OA:

Run order	Specific activity (U/mg protein)			Methionine uptake (%)	Dry weight (mg/ml)
	Actual value	Predicted value	Residual		
1	92	91.93	0.07	94.6	1.612
2	9.67	9.14	0.53	35.07	0.514
3	195.45	192.42	3.03	-	0.588
4	55.24	57.17	-1.93	-	1.208
5	9.31	7.61	1.70	15.98	0.94
6	33	41.31	-8.31	11.20	1.22
7	8.66	7.84	0.82	30.52	1.01
8	12.53	12.46	0.07	-	0.384
9	6.25	4.55	1.70	11.68	1.768
10	258.9	258.57	0.33	26.18	0.674
11	6.4	5.19	1.21	_	0.51
12	167.05	167.35	-0.30	15.88	0.998
13	31.23	30.02	1.21	36.68	1.202
14	15.26	18.57	-3.31	20.80	1.328
15	14.58	15.76	-1.18	24.32	0.462
16	23.125	22.80	0.33	42.18	0.44
17	53.75	54.43	-0.68	57.81	3.252
18	19.25	18.79	0.46	35.78	1.238
19	147.34	148.52	-1.18	61.72	2.388
20	10.53	11.21	-0.68	13.5	0.988
21	8.13	7.93	0.20	_	0.502
22	10.37	9.91	0.46	97.23	0.21
23	9.15	6.32	2.83	25.67	0.22
24	6.4	6.20	0.20	15.88	0.26
25	7.39	4.94	2.45	36.02	0.21

record for production L-methioninase by *Pythium* sp. and *Collectrichum* sp. While *C. globosum* has the best methioninase activity. Our results in this research directed to the identification of methioninlytic enzymes of the terrestrial fungus *C. globosum* as the first record. Thus, *C. globosum.* was selected as superior isolate for further experiment.

Morphological identification of *C. globosum*

C. globosum is a dematiaceous filamentous fungus isolated from soil, air and debris of plant. *C. globosum* is considered as causative agents of infections in humans. *C. globosum* have highly growth rate with texture is cottony; and color of surface colony is white and with colonies mature, color transform to olive while the color of reverse change from tan to red or brown to black. The fungal hyphae are septate, hyaline to brownish. Perithecia, asci and ascospores are present, the color of perithecia are brown to black, huge, brittle, and globose to flask-shaped and enclitic by tall spine. Perithecia have teeny rounded apertures named ostioles which include asci and ascospores inside ascospores are clavate to cylindrical in shape and are unicellular, brown in color (Ellis., 1971, 1976; Prokhorov and Linnik, 2011).

Sequencing of the 18S rRNA gene of *C. globosum*

Therefore, to portray the strain, the nucleotide sequences of the 18S rRNA of the strain were detected. Phylogenetic tree was structure by the method based on the 18S rRNA sequences. The 18S rRNA gene from the genomic DNA of the *C. globosum* (based on the Biochemical and staining characters) was enzymatically amplified by Taq DNA polymerase by using a universal fungal primer. From the phylogenetic analysis of sequence of *C. globosum* (Figure 1) with the watch closely related strains from the database. It appears a distinguished identity with *C. globosum.* The rRNA sequence of *C. globosum* was deposited to gene bank under accession number KXO24450 (http://www.ncbi.nlm.nih.gov/nuccore/KXO24450).

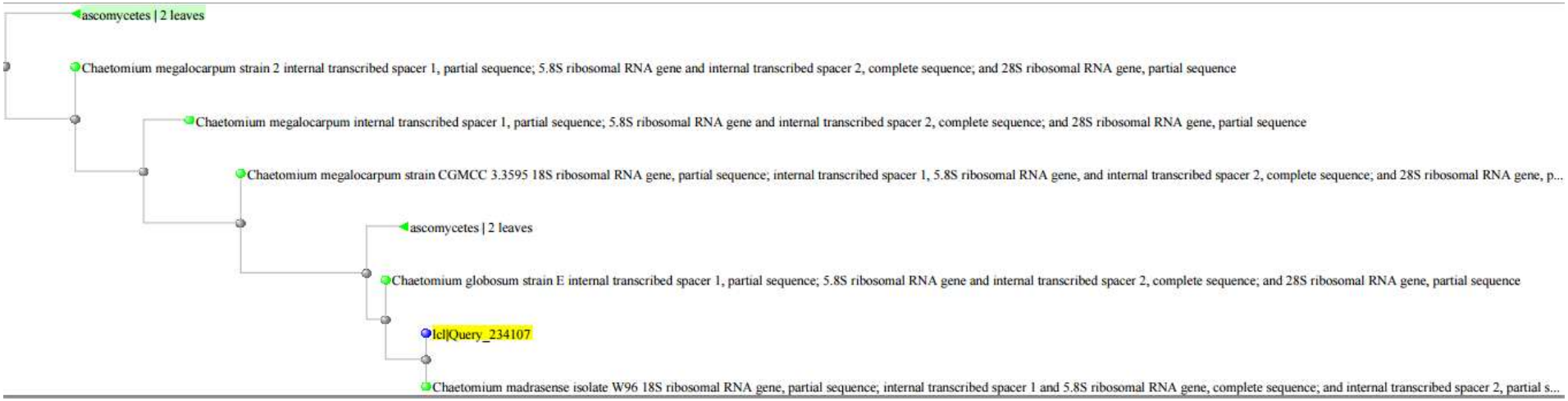


Figure 1. Phylogenetic analysis of *Chaetomium globosum.*

Taguchi orthogonal array (OA) experiments, statistical model and analysis

In Table 4, the ANOVA of L-methioninase production by *C. globosum* demonstrates that the model is significant due to a very high model F-value of 135.64 compared with a very low *p*-value of 0.0009. Values of "Prob > F" less than 0.05 point model terms are significant. For L-methioninase production model, B, C, D, E, F, AB, AC, AE, BC, BD, BE, BF, CD, CE, ABC, ABD, ABE, BCD are significant model terms. The model "Pred R-Squared" of 0.8126 is credible agreement with the model "Adj R-Squared" of 0.9916 with values close to 1 which shows good fit of the data to the regression model which also can be concluded from Table 2 due to small residuals between actual and predicted values.

The model "Adeq Precision"- measures the signal to noise ratio - of 42.646 shows an adequate signal and this model can be used to navigate the design space.

Last model equation for L-methioninase production by *C. globosum* in terms of coded factors:

L-Methioninase specific activity (U/mg protein) = -12.95 - 19.28*A - 594.02*B - 555.66*C -866.06*D - 587.41*E - 813.47*F - 1015.93*A*B - 1366.78*A*C + 42.97*A*D - 1077.11*A*E - 224.81*B*C - 128.49*B*D - 257.23*B*E - 905.15*B*F + 935.84*C*D + 183.16*C*E - 125.18*A*B*C - 82.41*A*B*D - 246.19*A*B*E - 37.35*A*B*F - 91.86*B*C*D

Where, A = Incubation time (days); B = Temperature (ºC); C = pH; D = Methionine concentration (%); E = Sucrose (%); F = Sodium nitrate (%).

The three dimensional (3D) response surface plots-generated by Design-Expert software shown in Figure 2 represent the relationships and effects of different experimental variables (factors) on L-methioninase productivity produced by *C. globosum*. Best experimental variables levels for maximizing L-methioninase production were predicted through analysis of these plots in combination with numerical optimization for each variable and desirability analysis.

Optimum conditions for L-methioninase production

According to desirability analysis of the model variables and numerical optimization, the optimum levels of the incubation period, temperature, pH, methionine, sucrose and sodium nitrate concentrations were 3 days, 30°C, 7, 0%, 30 g/l and 1 g/l, respectively. According to the produced model, at these levels, *C. globosum* produce L-methioninase with predicted specific activity of

Table 4. Analysis of variance (ANOVA) for response surface to reduce cubic model for L-methioninase production by *Chaetomium globosum.*

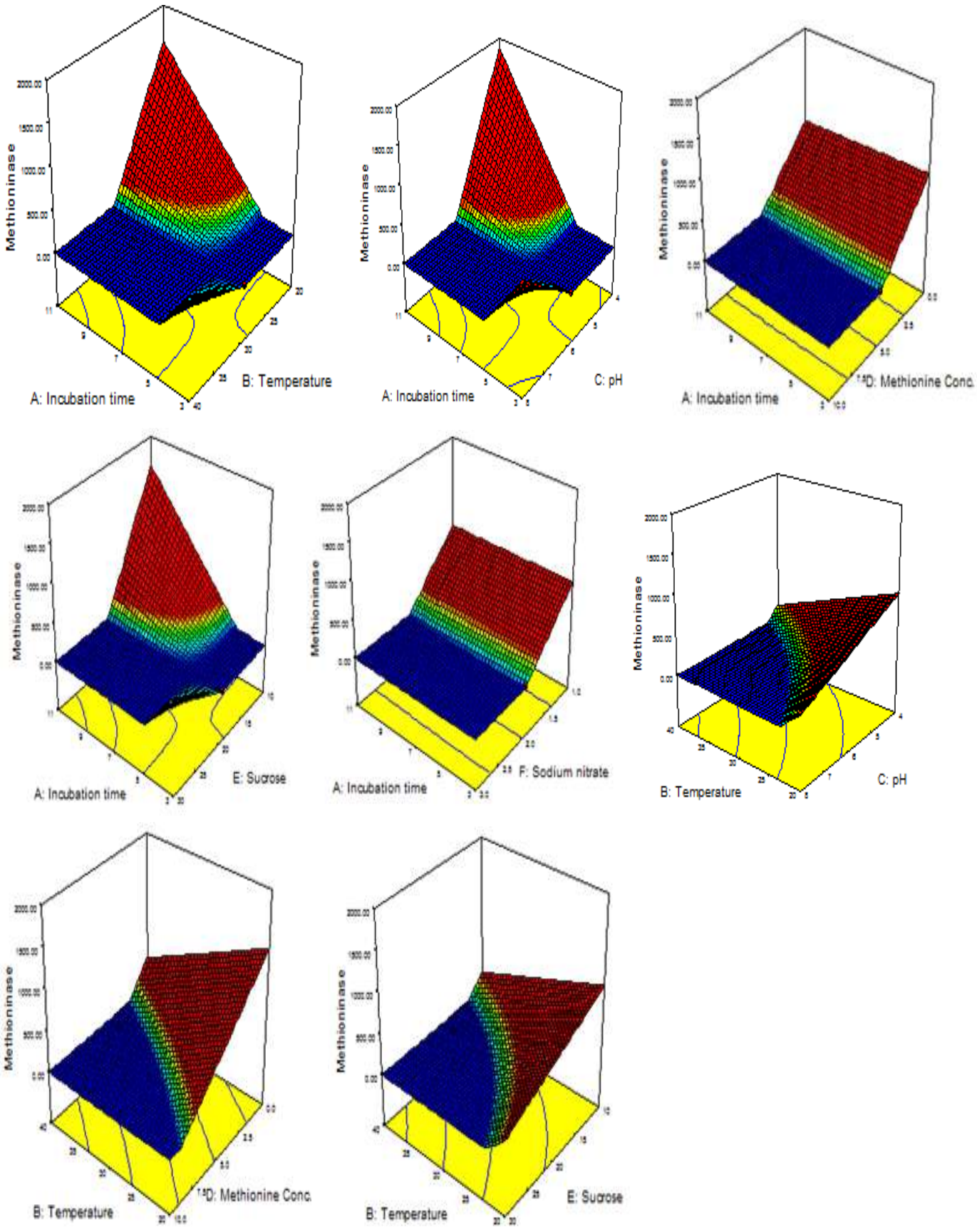
Source	Sum of squares	df	Mean square	F-Value	*p*-Value Prob > F*
Model	114841.90	21	5468.66	135.64	0.0009
A-Incubation time	402.18	1	402.18	9.98	0.0509
B-Temperature	5804.92	1	5804.92	143.98	0.0012
C-pH	6038.95	1	6038.95	149.78	0.0012
D-Methionine concentration	5658.72	1	5658.72	140.35	0.0013
E-Sucrose	5565.11	1	5565.11	138.03	0.0013
F-Sodium nitrate	5412.53	1	5412.53	134.24	0.0014
AB	5143.26	1	5143.26	127.57	0.0015
AC	5581.20	1	5581.20	138.43	0.0013
AD	346.03	1	346.03	8.58	0.0610
AE	6152.06	1	6152.06	152.59	0.0011
BC	3638.35	1	3638.35	90.24	0.0025
BD	5450.18	1	5450.18	135.18	0.0014
BE	3750.80	1	3750.80	93.03	0.0024
BF	5036.00	1	5036.00	124.91	0.0015
CD	5460.29	1	5460.29	135.43	0.0014
CE	5606.13	1	5606.13	139.05	0.0013
ABC	2381.47	1	2381.47	59.07	0.0046
ABD	1480.04	1	1480.04	36.71	0.0090
ABE	13740.96	1	13740.96	340.81	0.0003
ABF	315.07	1	315.07	7.81	0.0681
BCD	2830.56	1	2830.56	70.21	0.0036
Residual	120.95	3	40.32		
Cor Total	114962.86	24			

*Values of "Prob > F" less than 0.05 indicate model terms are significant. Reduced cubic model.

(≈2225 U/mg). The L-methioninase production by *Antechinus flavipes* is similar to the L-methioninase produced by *Achromobacter starkeyi, Aspergillus* sp. RS-1a, *Phoronis ovalis* and *Yarrwia lipolytica* (Ruiz-Herrera and Starkey, 1970; Rifai, 1969; Tanaka et al., 1976; Bondar et al., 2005) found to be L-methionine dependent. In contrast, L-methioninase biosynthesis by *Geotrichum candidum* and *Pseudomonas putida* were found to be L-methionine freelance (Bonnarme et al., 2001; Tan et al., 1997). The effect of various L-methionine concentrations on enzyme productivity by *A. flavipes* was investigated. The initial concentration of fermentation medium L-methionine does a significant influence on the uptake of L-methionine and so, on enzyme productivity by *A. flavipes*. The highest yield of L-methioninase and methionine uptake (94%) by *A. flavipes* was registered using 0.8% L-methionine. Higher levels of L-methionine (3.2%) suppress the enzyme yield by about 42.5% on a par with the control. It could be finished that the productivity of L-methioninase by the fungal isolate is L-methionine concentration subordinate. Moreover, the growth rate of *A. flavipes* was gradually raised with the level of L-methionine, arriving to its highest value (6 g/L) at 0.8% L-methionine, pursued by a gradual reduced to about 41.7 at 3.2% L-methionine. The lower enzyme yield with higher concentrations of L-methionine may be imputed to the down regulation of GATA gene transcription that blocked the gene expression of methioninase (Caddick et al., 1994, Mitchell and Magasanik, 1984), methionine catabolic suppression, or the transinhibition phenomenon (Pall, 1971).

The production of L-methioninase in any case the presence of its inducer methionine in the culture medium suggestes that, L-methioninase was found to be L-methionine freelance. Similar results were denoted for L-methioninase production by *G. candidum* and *P. putida* ((Bonnarme et al., 2001; Tan et al., 1997).

In contrast, Khalaf and El-Sayed (2009) indicated that the fashioning of L-methioninase production depended on L-methionine on containing medium (Bonnarme et al., 2001) on *Yarrwia lipolytica*. In addition to, the use of yeast extract and peptone in medium for the production of L- methioninase was registered by Arfi et al. (2006) from *G. candidum*.


Methioninase
A: Incubation time
B: Temperature
C: pH
D: Methionine Conc.
E: Sucrose
F: Sodium nitrate

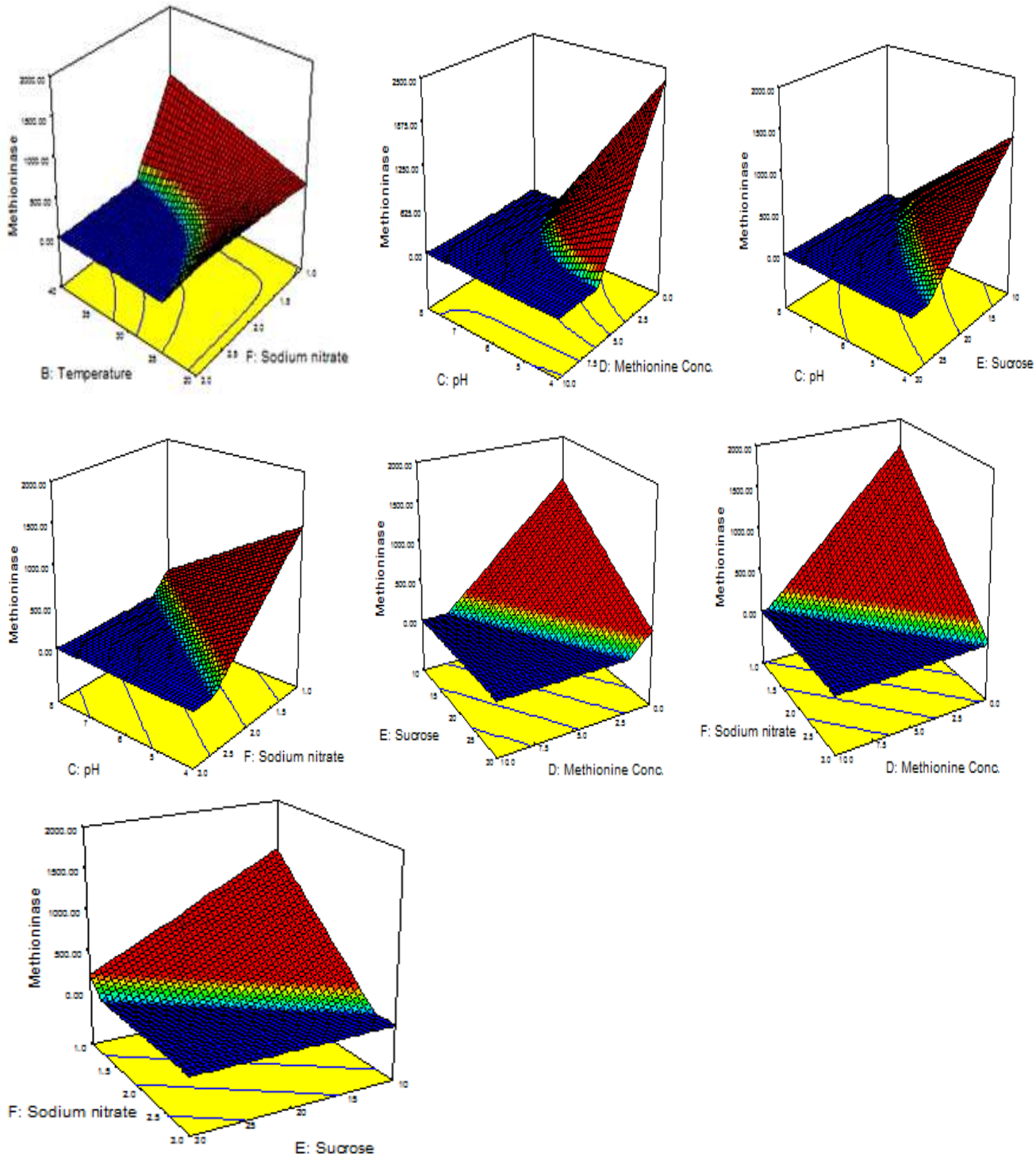


Figure 2. Response surface plots showing the effect of incubation time, temperature, pH, methionine concentration, sucrose concentration and sodium nitrate concentration for production of L-methioninase in terms of specific activity (U/mg).

Conclusion

In this study, the production of L-Methioninase (E.C 4.4.1.11) from a novel fungal source (*C. globosum*) from Egyptian soil as well as statistical modelling using AO, of the production process based on the production

Variables was successful. L-methioninase was also produced from fungal source at low methionine concentration and used in biotechnology and medical application.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Isolation and identification of potential probiotic bacteria on surfaces of *Oreochromis niloticus* and *Clarias gariepinus* from around Kampala, Uganda

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Increased fish mortality due to infections has forced most farmers to resort the use of chemotherapeutic agents especially antibiotics. The continued use of these drugs in aquaculture is becoming limited as pathogens develop resistance and infer unpredicted long term public health effects. More research efforts are building to identify alternative disease prevention methods, among which the use of probiotics has been proposed. Therefore, the purpose of this study was to identify potential probiotics on surfaces of tilapia and catfish in areas around Kampala. Tilapia and catfish samples were aseptically collected from selected cages, ponds, tanks and hatcheries around Kampala, including Lake Victoria. The skin of fish was swabbed and then cultured on both general purpose and selective media. Probiotic screening was done using the agar spot method. Results revealed complete growth across all samples. The total microbial load was highest in fish from lakes ($1000\pm9.6\times10^5$ cfu) and cages ($1001\pm5.0\times10^5$ cfu). In all cases tilapia fish was significantly ($p<0.0001$) more contaminated than catfish. Out of the three strains of probiotics isolated, only *Lactobacillus spp* and *Lactococcus spp* showed antibacterial activity against pathogenic bacteria. The activity of *Lactobacillus spp* was significantly high ($p< 0.0001$) with *Streptococcus spp* (16.5±0.2 mm). *Lactobacillus spp* inhibited growth of only *Proteus spp* (5±0.2 mm). Our study shows that *Lactobacillus spp* and *Lactococcus spp* isolated from tilapia and catfish possess probiotic activity against a number of pathogenic bacteria. Our findings have significant implications for subsequent probiotic formulation and testing in aquaculture.

Key words: Probiotics, *Oreochromis niloticus*, *Clarias gariepinus*, aquaculture, Uganda.

INTRODUCTION

According to the Food and Agriculture Organization of the United Nations, presently 52% of the 600 wild fish species with economic value are threatened (Dudgeon et al., 2006), 17% over fished and 7% fully exploited (Naylor et al., 2000). In Uganda, the fish industry greatly contributes to the welfare of Ugandans in terms of employment, food security, government revenue and foreign exchange earnings (Nyombi and Bolwig, 2004). The

main fish species on the Ugandan market include; Nile perch (*Lates niloticus*), Nile tilapia (*Oreochromis niloticus*), and catfish (*Clarias gariepinus*) (Kabahenda and Hüsken, 2009). Aquaculture is currently the fastest growing food production sector in the world, expanding the total world production and diversity of cultured species (Naylor et al., 2000). The global production of fish from capture fisheries and aquaculture increased by 7.5 to 59.9% in 2010 from 55.7% in 2009 (Esteban et al., 2013).

Aquaculture is an important sector in Uganda providing alternative employment opportunities but fish diseases especially bacterial infections remain primary constraints to its continued expansion (Austin and Austin, 2007; Tellez-Bañuelos et al., 2010). In contrast to the intestines, little is known about the development or activity of bacterial flora on gills and surfaces of fish. Stress weakness is the fish's natural mechanism of defense, making it more susceptible to disease (Plumb and Hanson, 2011). With the growing concern of diseases, most farmers especially in shrimp farming have turned to antimicrobial drugs to cure the bacterial infections (Holmström et al., 2003). Although antibiotics improve survival, they also alter the microbial communities and induce resistant bacteria populations, with unpredictable long term effects on public health (Luis Balcázar et al., 2006). The use of antibiotics to cure bacterial infection and prevent fish mortality in aquaculture is becoming limited as pathogens develop resistance to the drugs and accumulation of antibiotic residues in fish tissues (De La Peña and Espinosa-Mansilla, 2009).

Furthermore, beneficial bacterial flora are killed by antibiotic administration, leading to more efforts to find alternative disease prevention methods such as use of nonpathogenic bacteria called probiotics (Kesarcodi-Watson et al., 2008). Probiotics are beneficial microorganisms with ability to reduce the use of antibiotics in aquaculture since their addition can assist in returning a disturbed microbiota to its normal beneficial composition (Defoirdt et al., 2011). According to Verschuere et al. (2000), the interaction between the probiotics and the host is, however not limited to the intestinal tract but also on the surfaces of skin and gills of the fish and its ambient environment. The majority of identified probiotics in fish belong to the lactic acid bacteria (*Lactobacillus*), *Vibrio*, *Bacillus* and *Pseudomonas* genera's (Gatesoupe, 1999). A number of commercially formulated probiotics are now being utilized in aquaculture but with mixed success results. Therefore, it is likely that for probiotics to be effective, they need to be isolated from the same environment where the fish is farmed. Therefore, the aim of the current study was to isolate and identify potential probiotic bacteria on the surface of Nile Tilapia (*Oreochromis niloticus*) and Catfish (*Clarias gariepinus*) from different production systems around Kampala. Results from this study will form a basis for the production of probiotic formulations and subsequent testing in aquaculture.

MATERIALS AND METHODS

Study design

A total of 45 Nile Tilapia (*Oreochromis niloticus*) and 45 Catfish (*Clarias gariepinus*) were purposively collected from Mulungu Island in Lake Victoria, hatcheries (fingerlings) in Kawempe, ponds and tanks at Kajjansi Research Institute and fish cages at Kitinda, all around Kampala district. Fish from the lake and ponds were captured using a cast net, with each fish put in a separate plastic bag and immediately transported to the Microbiology Laboratory at the College of Veterinary Medicine, Animal Resources and Biosecurity in iceboxes. Only freshly captured fish samples were included in the study, dead fish at capture were excluded. In the Laboratory, the skin of both catfish and tilapia were aseptically swabbed with subsequent culturing on sterilized media plates. Plates were incubated at 37°C for 24 h. Isolates were sub cultured to obtain pure cultures that were further identified using gram staining and biochemical tests.

Bacteria isolation and identification

Bacteriological media: Nutrient agar, MacConkey agar, Potato Dextrose Agar (PDA), de Man, Rogosa and Sharpe agar (MRS), Mannitol Salt Agar (MSA), Thiosulfate-citrate-bile salts-sucrose agar (TCBS) and Blood agar were prepared according to manufacturer's instructions (Sigma-Aldrich, USA). The media were sterilized at 121°C for 15 min in an autoclave and later poured into sterilized disposable plastic petri dishes. The petri dishes were then stored in the incubator after media drying. A sterile cotton swab was brushed all over the skin of the fish. Swabs were then swirled into sterilized peptone water that was serially diluted into five dilutions of 9ml. Bacterial cultures followed the method as described by Boone et al. (2001). Briefly, a quantity of 0.1ml of 10^{3} and 10^{-5} dilution was inoculated in Petri dishes of Nutrient Agar, MacConkey agar, TCB, PDA, MSA agar plates in duplicates and spread using a sterile glass rod, then incubated aerobically for 24 to 48 h at 37°C and anaerobically for MRS agar plates at the same temperature and hours.

Colony count was calculated by dividing the bottom of the Petri dish into four and the sum of bacterial count was multiplied by the dilution factor. Each distinct colony was further sub cultured on freshly prepared Nutrient agar for evaluation of purity and colonial morphology. The isolates were further subjected to Gram stain to determine their Gram reaction and biochemical test as described by (Cheesbrough, 2006) and (Mac Faddin, 1976) and also, to determine the identity of bacteria isolates.

Antimicrobial activity

The probiotic strains were screened for antimicrobial activity against selected pathogens using an agar spot method as described by (Schillinger and Lücke, 1989). Briefly, overnight cultures of

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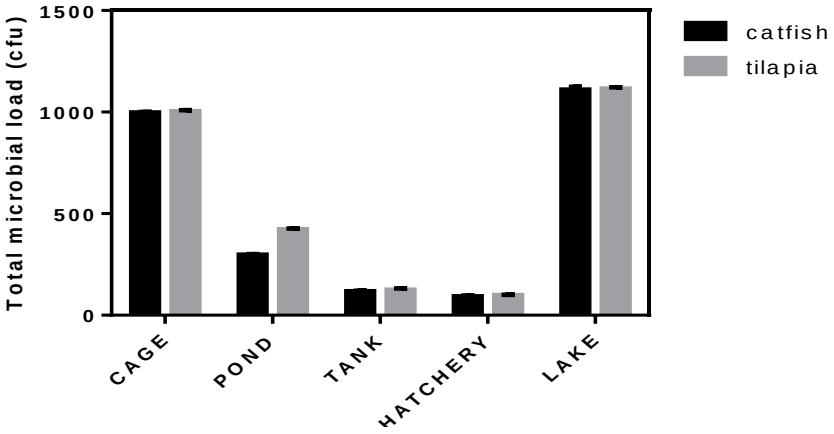

Figure 1. Total microbial load on surfaces of tilapia and catfish from selected sampling systems.

Lactobacillus spp, Bacillus sabtillis and *Lactococcus spp* were spotted onto the surface of MRS agar (1.2% w/v agar, 0.2% w/v glucose) plates, which were then incubated anaerobically for 24 h at 37°C.The indicator species (*Staphylococcus aureus, Streptococcus spp, Proteus spp and Pseudomonas spp*) were inoculated into 7ml of soft agar medium (nutrient broth containing 0.7% w/v) to a final concentration of approximately 10^5cfu. The soft media were later poured on the plates and incubated for 24 h at 37°C. Zones of clearance were later measured in mile meters.

Statistical analyses

Statistical analyses were done using Graph pad 6.0 statistical software. Total microbial load across sampling sites and fish species were done using a Two-way ANOVA. Significant differences in antibacterial activity across the different pathogenic bacteria were analyzed using a One-way ANOVA set at significance level of ($p< 0.05$). Multiple comparisons between groups (sampling sites and pathogenic bacteria strains) were done using Tukey`s multiple comparison test, differences were taken as significant at $p< 0.05$.

RESULTS

Total microbial load

The results revealed that the sampling site and fish type had a significant effect on total microbial load ($p< 0.0001$, $F_{(4,10)} =72.15$, $P> 0.0001$, $F_{(1,10)} =111.1$) respectively. On comparison between sampling sites, microbial load on fish surfaces was significantly higher ($p<0.05$) in lakes ($1000\pm9.6\times10^5$ cfu) and cages ($1001\pm5.0\times10^5$ cfu) as compared to tanks ($121.6\pm6.3\times10^5$ cfu), ponds ($360.5\pm72.2\times10^5$ cfu) and hatcheries ($90\pm4.3\times10^5$ cfu) (Figure 1). Hatcheries and tanks had the least microbial load. On comparison between tilapia and catfish, microbial load in tilapia from ponds was significantly higher ($p< 0.05$) as compared to catfish from the same source. No significant difference ($p>0.05$) between the two fish species were observed across the other sampling localities.

Prevalent bacteria isolated on surfaces of tilapia and catfish

The data showed that the most commonly isolated bacteria on the surface of tilapia across sampling systems were: *Escherichia coli* (82%), *Lactococcus spp* (80%), *Staphylococcus aureus* (73%), *Streptococcus spp* (69%), *Proteus spp* (60%), *Lactobacillus spp* (48%) and *Klebsiella spp* (47%). The least isolated being *Pseudomonas spp* (38%), *Bacillus subtillis* (27%), *Corynebacteria spp* (24%), *Bacillus cereus* (20%), and *Enterobacteria spp* (16%) (Table 1). For catfish, the most commonly isolated bacteria on the surface across the sampling systems were*: Lactococcus spp* (87%), *Escherichia coli* (80%), *Staphylococcus aureus* (64%), *Streptococcus spp* (60%), *Lactobacillus spp* (60%) *Proteus* spp (53%) *and Klebsiella* spp (47%) (Table 2).

Antibacterial activity of selected probiotic genera

When antibacterial activity as a measure of probiotic potential was determined, only two genera (*Lactobacillus and Lactococcus)* showed probiotic potential. The results revealed that antibacterial activity significantly varied ($p<0.05$) across the different pathogenic bacteria utilized. When antibacterial activity was compared across the different pathogenic isolates, *Lactobacillus spp* had a significantly ($p<0.0001$) higher activity. *Lactobacillus spp* showed the highest activity on *Streptococcus spp* (16 ± 0.2mm), compared to *Proteus spp* (9 ± 0.2 mm) and *Pseudomonas spp* (7 ± 0.2mm, Figure 2A). Antibacterial activity of *Lactococcus spp* was observed for only *Proteus spp* (5 ± 0.2mm, $p< 0.0002$) (Figure 2B). No probiotic potential was observed for *Bacillus subtillis.*

Table 1. Bacteria isolated on surfaces of tilapia.

Bacteria	Cage	Pond	Hatchery	Tank	Lake	Total
Eschericchia coli	8(80%)	7(70%)	6(60%)	7(70%)	9(90%)	37(82%)
Lactococcus spp	8(80%)	7(70%)	6(60%)	5(50%)	10(100%)	36(80%)
Staphylococcus. auerus	9(90%)	7(70%)	2(40%)	5(50%)	10(100%)	33(73%)
Streptococcus spp	10(100%)	8(80%)	0	4(40%)	9(90%)	31(69%)
Proteus spp	8(80%)	7(70%)	0	4(40%)	8(80%)	27(60%)
Lactobacillus spp	5(50%)	3(30%)	4(40%)	4(40%)	6(60%)	22(48%)
Klebsiella spp	7(70%)	5(50%)	1(20%)	2(20%)	6(60%)	21(47%)
Pseudomonas spp	7(70%)	0	0	3(30%)	7(70%)	17(38%)
Bacillus spp	2(20%)	2(20%)	5(50%)	0	3(30%)	12(27%)
Corynebacteria spp	5(50%)	0	0	0	6(60%)	11(24%)
Bacillus cereus	0	4(40%)	0	0	5(50%)	9(20%)
Enterobacteria spp	6(60%)	5(50%)	1(20%)	2(20%)	6(60%)	7(16%)

Table 2. Bacteria isolated on surface of catfish.

Bacteria	Cage	Pond	Hatchery	Tank	Lake	Total
Lactococcus spp	9(90%)	8(80%)	7(70%)	5(100%)	10(100%)	39(87%)
Escherichia coli	7(70%)	5(50%)	5(50%)	6(60%)	8(80%)	36(80%)
Staphylococcus aureus	8(80%)	6(60%)	1(20%)	4(40%)	10(100%)	29(64%)
Streptococcus spp	9(90%)	7(70%)	0	3(30%)	8(80%)	27(60%)
Lactobacillus spp	6(60%)	4(40%)	5(50%)	5(100%)	7(70%)	27(60%)
Proteus spp	7(70%)	6(60%)	0	3(30%)	8(80%)	24(53%)
Klebsiella spp	6(60%)	4(40%)	1(20%)	4(40%)	7(70%)	21(47%)
Bacillus subtillis	3(30%)	3(30%)	6(60%)	0	4(40%)	16(36%)
Pseudomonas spp	6(60%)	0	0	2(20%)	6(60%)	14(31%)
Enterobacteria spp	5(50%)	5(50%)	1(20%)	0	0	11(24%)
Corynebacteria spp	4(40%)	0	0	0	5(50%)	9(20%)
Bacillus cereus	0	3(30%)	0	0	4(40%)	7(16%)

DISCUSSION

There has been a growing concerns about the adverse effects of bacterial diseases in aquaculture of many economically important marine and fresh fish species including Nile tilapia and Catfish (Ashley, 2007). Bacterial infections cause considerable losses to the fish industry especially from mortality and reduced growth (Austin and Austin, 2007), forcing most farmers to resort to use of chemotherapeutic agents especially antibiotics. The continued use of these

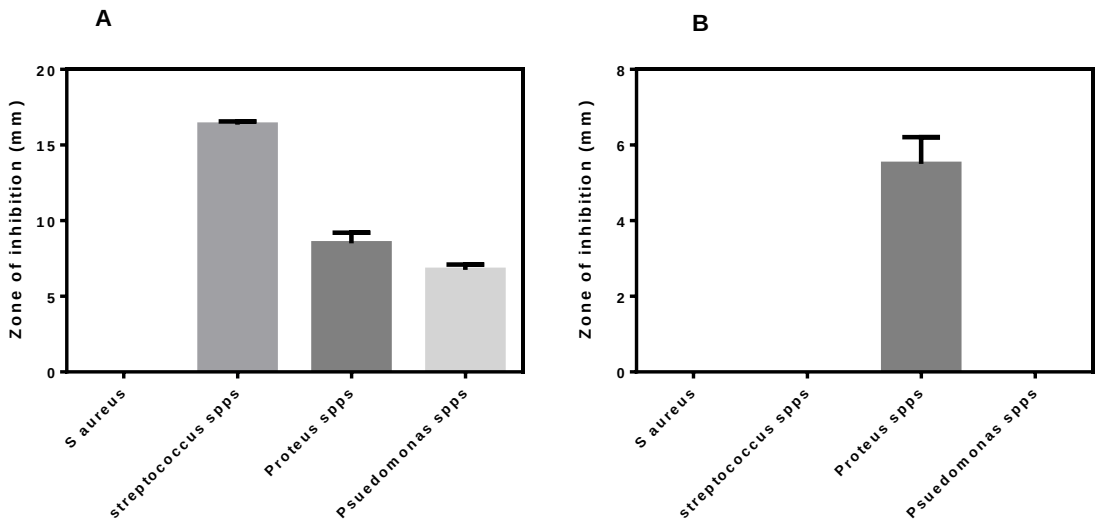

Figure 2. Probiotic activity of *Lactobacillus spp* (A) and *Lactococcus spp (B)* on selected pathogenic bacteria.

drugs in aquaculture has become limited as pathogens developed resistance to drugs (Alderman and Hastings, 1998). Probiotics have been proposed as possible alternatives to the use of antibiotics (Joerger, 2003). Therefore, the purpose of this study was to isolate and identify potential probiotic organisms on surfaces of tilapia and catfish from areas around Kampala district, Uganda.

In this study, the total microbial load was significantly high on both surfaces of tilapia and catfish from lakes ($1000\pm9.6\times10^{-5}$ cfu) and cages ($1001\pm5.0\times10^{-5}$ cfu). We further showed that microbial load in tilapia from ponds was significantly higher as compared to catfish from the same source. Total microbial load for both catfish and tilapia in this study was slightly higher compared to that reported by (Emikpe et al., 2011) in catfish ($117.33\times10^{-}$11cfu) and tilapia ($143.67\times10^{-}$11cfu). Generally, increase in total microbial load has been attributed to high aquatic temperatures resulting from organic matter recycling, self-cleaning potential, and re-mineralization (Fernandes et al., 1997; Hossain et al., 1999). Variations in bacterial counts between individual fish have been observed previously (Spanggaard et al., 2000) and were confirmed by our results.

The present study revealed 12 genera of bacteria on the surfaces of both catfish and tilapia from various aquatic environments. These results were in agreement with the findings of (Adebayo-Tayo et al., 2012). Some of the bacteria species recovered in this study were also identified from healthy *Clarias gariepinus* (Efuntoye et al., 2012). The presence of these isolated organisms was not surprising since fish live in water habitat full of micro-organism. Among these isolates, *Escherichia coli* were the most dominant in both catfish and tilapia. Increased presence of *E. coli* might demonstrate the level of habitat pollution because coliforms are not the normal flora of bacteria in fish (Mandal et al., 2009). Similarly, like in this study, other studies such as Ibrahim and Sheshi (2014) have demonstrated the presence of *Staphylococcus aureus*, which also less frequently occurs as natural microflora of fish.

From this study, three proposed probiotic genera were isolated; *Lactobacillus spp, Lactococcus spp* and *Bacillus* subtillis. These strains were similarly isolated from the gut of the Nile tilapia (Zapata and Lara-Flores, 2012). The findings are in agreement with (Ringø et al., 1997) who found that 10% microbiota population in Artic charr (*Salvelinus aplinus L.)* was lactic acid bacteria. The findings of our study also confirm a study by (Hamid et al., 2014) who isolated *Lactococcus spp and Lactobacillus spp* from catfish. However, reports on the presence of *Lactococcus spp* in freshwater fishes are scarce. In the present study, as reported previously by Einar Ringø and Gatesoupe (1998), *Lactobacillus spp* had the highest antimicrobial activity against all the selected pathogens tested except for *Staphylococcus spp*. Its activity was highest against *Streptococcus spp* (16±0.2mm), followed by *Proteus spp* (9 ± 0.2mm) and least for *Pseudomonas spp* (7 ± 0.2mm). The mechanism of antibacterial activity in *Lactobacillus* strains appears to be multifactorial (Servin, 2004). Ali et al. (2013) revealed that all lactobacilli tested (except *L. delbrueckii*) inhibited the growth of *S. aureus*. This probiotic activity of *Lactobacillus spp* on pathogenic bacteria has already been demonstrated in a number of studies in fish (Kim et al., 2007; Nayak, 2010; Nikoskelainen et al., 2001; Suzer et al., 2008). In our study, *Lactococcus spp* showed antimicrobial activity only against *Proteus spp* (5 ± 0.2mm). In another study, *Lactococcus spp* was reported to inhibit the fish pathogen, *Aeromonas hydrphila* in tilapia (Hamid et al., 2014).

In our study, surprisingly *Bacillus subtillis* isolated from

fish surfaces did not show any antimicrobial activity against the selected pathogenic bacteria. According to (Domrongpokkaphan and Wanchaitanawong, 2006) *Bacillus subtillis* isolated from hepato-pancreas of black tiger shrimp were found active against four shrimp pathogenic *Vibrio spp.* Indeed, (Sugita et al., 1998) observed *Bacillus subtillis* from fish gut to produce antibacterial substances. It is difficult to comment on the reason for this variability in antimicrobial activity amongst isolates from different fish anatomical compartments. However, as stated by Jacobsen et al. (1999) it is likely that the environment from which the bacteria are isolated might have a role in determining probiotic potential.

Conclusion

In conclusion, our study shows that total microbial load was highest in both tilapia and catfish sampled from cages and lakes compared to fish species from ponds, tanks and hatcheries.

The most commonly isolated potentially pathogenic organisms on both surfaces of catfish and tilapia included; *Escherichia coli, Staphylococcus aureus, Streptococcus spp and Proteus spp, Klebsiella spp* and *Pseudomonas spp.* Three probiotic species: *Lactococcus spp, Lactobacillus spp* and *Bacillus spp* were isolated. *Lactobacillus spp* showed the highest antimicrobial activity followed by *Lactococcus spp. Bacillus subtillis* showed no antimicrobial activity against selected pathogenic isolates.

However, future studies characterizing the observed probiotic species would be important to aid their use in aquaculture. Furthermore, studies evaluating probiotic potential using a combination of two or more organisms would be important for improved activity.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Rapid microbiological tests for prospecting new fungal strains with high potentiality for the pectinolytic enzymes production

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Prospecting of new isolates producers of pectinolitic enzymes were performed with cultivation of substrates of rice, soybean and corn bran. Pectinolitic enzymes were observed in cultures of the isolates NFC 1, NFC 2, NFC 4, NFC 5 and NFC 6 identified as belonging to the genus *Aspergillus* sp. Isolates NFR 1, NFR 2 and NFS1 were identified as belonging to the genus *Rhizopus* and NFC 3 belonging to the genus *Cladosporium*; however, pectinolitic enzymes production was not observed. The polygalacturonase (PG) production in submerged fermentation of *Aspergillus* NFC 2 in culture medium proposed (SM) at 28 and 37°C was quantified by agar diffusion and spectrophotometric methods. The highest PG production was obtained at 28°C by greater formation of halo degradation pectin and the activity measured by spectrophotometric methods. The activity of PG obtained was 7 U.mL^{-1} with volumetric productivity greater in the first 24 h of fermentation both at 28 and 37°C. A decrease in PG synthesis occurred after 48 to 120 h. The study allowed, by means of rapid microbiological tests, the selection of new isolates fungi for PG production and the definition of crucial stages for cultivation in liquid medium.

Key words: Filamentous fungi, *Aspergillus* sp, pectinolytic enzymes, polygalacturonase, submerged fermentation.

INTRODUCTION

The search for new biomolecules requires the isolation of microorganisms and the understanding of its genetic heterogeneity and metabolic (García and Bianchi, 2015; Pedrosa et al., 2013). The fungi are considered as promising sources of new biomolecules and have been employed for therapeutic use (Strobel and Daisy, 2003). These microorganisms represent an important genetic source for biotechnology, having stimulated the interest

of the scientific community due to the production of biomolecules with applications, mainly in the food and pharmaceutical industry (Strobel and Daisy, 2003). There are more than 50,000 secondary metabolites obtained by culture of microorganisms, being that, 12,000 are known antibiotics. Of these, 55% were produced by actinomycetes and 22% by filamentous fungi (Demain, 1999). Brazil offers a wide biodiversity, having a wide field to be explored in search of promising microorganisms in the production of biomolecules. The Tocantins State is located in the geographical zone of transition between the cerrado and the Amazon forest, and in both biomes, are characterized for being potential sources of microorganisms (Silva et al., 2015).

Microbial enzymes are widely used in industrial processes possessing a wide application in the conversion of food, in pharmaceutical production of medicines, leather industry, detergents, textiles and in the management of industrial waste (Lima, 2014), may be obtained by bacteria, yeasts and mainly by filamentous fungi from genus *Aspergillus* that are capable of producing enzymes in culture medium of low cost, secreting extracellularly. These fungi present formation of septate and branched mycelium, white or yellowish coloration with formation of stalks (Guimarães et al., 2006; Lelis et al., 2012; Haas et al., 2013). Pectinase production on an industrial scale by *Aspergillus* sp. presents advantage by high capacity for conversion with around 90% of products formed during fermentation (Fontana and Silveira, 2012; Sandri et al., 2015).

The pectinases are typical examples of enzymatic groups with wide applicability in the market, because they have demand and properties that meet the technical and economical requirements in industrial scale (Rehman et al., 2016). The classification of this group of enzymes is based on enzymatic attack to galacturonic skeleton; in preference for a particular substrate; in the mode of action by hydrolysis and in the cleavage of the substrate, random or terminal (Carvalho et al., 2013; Kant et al., 2013).

The polygalacturonase (EC 3.2.1.15) is pointed out as the main hydrolase enzyme and is classified as a result of its action mechanism on the substrate in two groups: Endo-polygalacturonase and exo-polygalacturonase (Pan et al., 2015; Rehman et al., 2012). This enzyme is commonly produced by *Aspergillus*, *Agrobacterium*, *Aureobasidium*, *Bacillus, Colletotrichum*, *Clostridium*, *Fusarium, Geotrichum, Penicillium, Rhizopus*, *Saccharomyces* and *Trichoderma* (Favela-Torres et al., 2008; Fontana and Silveira, 2012; Lelis et al., 2012; Sandri et al., 2015).

Thus, the present work has the objective of prospecting new fungal isolates from cereals originated in the geographical zone of transition between the cerrado and the Brazilian Amazon forest and the characterization of species by means of rapid microbiological tests that comprise potential in the PG production before complete identification. Besides, this work contributed to definition of crucial stages for cultivation in liquid medium.

MATERIALS AND METHODS

Microorganisms, production of inoculum and culture conditions

The microorganisms tested for production of pectinolitic enzymes were filamentous fungi isolated from rice (NFR), soybean (NFS) and corn bran (NFC) obtained from cereals originating from the geographical zone of transition between the cerrado and the Brazilian Amazon forest, Gurupi city, Tocantins, Brasil. The prospecting of new fungi from cereals was based on the possibility of application in animal feeding. Coordinates study was 11° 43' 45" S, 49° 04' 07" W.

All the cereals were packaged in closed containers with 100 g of each and soaked with 100 mL of nutrient solution containing 120 $g.L^{-1}$ sucrose and 30 $g.l^{-1}$ of NaCl. The flasks soaked with nutrient solution were incubated for 5 days at 25°C. Sample of cultivation of each substrate was transferred in potato dextrose agar medium (PDA) and incubated at 25°C for five (5) days. In this step, ten (10) fungi isolates were obtained. After isolation, the bank of the culture of each fungi isolate was prepared and stored in ultra-freezer at 80°C (Sanyo - VIP vertical 519L). In all the experiments for PG production, the activation of fungi occurred in PDA medium at 25°C for three (3) days. This work used *Aspergillus parasiticus* as positive control for PG production. This reference fungi was provided by Integrated Pest Management Laboratory of the Federal University of Tocantins.

Reproductive structures and morphological aspects

The identification of fungi isolates was performed after cultivation on PDA culture medium. Sample of spores was fixed on slides and analyzed in binocular optical microscope (Nikon, Eclipse E200). Analyzes of the structures of spores and septa mycelium were observed with increase at 1000X. After analysis, the classification of each fungi isolate was performed to identify up to genus (Griffin 1994).

The submerged fermentation

A new culture medium was proposed for cultivation in submerged fermentation (SM) composed of 2 $g.l^{-1}$ of calcium chloride; 5 $g.l^{-1}$ of ammonium nitrate; 0.5 $g.l^{-1}$ of potassium chloride; 0.02 $g.l^{-1}$ of magnesium sulphate; 0.02 $g.l^{-1}$ of iron sulphate; 20 $g.l^{-1}$ of glucose; 10 $g.l^{-1}$ of pectin; 7.5 $g.l^{-1}$ of yeast extract and 2 $g.l^{-1}$ of peptone of meat. The effects of temperature on the production of PG were

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evaluated with cultivation at 28 and 37°C for five (5) days. Supernatant of the samples were analyzed by measure of the halo degradation pectin and the activity of PG production by spectrophotometric methods.

Qualitative test of pectinolitic enzymes

The qualitative test of PG production was performed by means of culture in agar diffusion with evaluation of halo formation of pectin degradation. The culture medium in agar diffusion was composed of 1 $g.l^{-1}$ of corn bran; 2 $g.l^{-1}$ of citrus pectin; 2.2 $g.l^{-1}$ glucose; 0.005 $g.l^{-1}$ yeast extract; 0.5 $g.l^{-1}$ iron sulphate and ammonium; 0.05 $g.l^{-1}$ of magnesium sulphate; 0.25 $g.l^{-1}$ of monopotassium phosphate ; 62 $\mu g.l^{-1}$ of zinc sulphate; 1 $\mu g.l^{-1}$ of sulphate manganese and 2 $g.l^{-1}$ of agar. The pH was adjusted to pH 4 (Fontana and Silveira, 2012). After activation of fungal isolates on PDA culture medium, samples of crops were evaluated in agar diffusion to 28°C observing the formation of halo of pectin degradation. In the first step of fermentation, submerged samples were evaluated in agar diffusion at 28 and 37°C observing the formation of halo degradation pectin. The center point of the plate was determined as a referential for the measurement of the halos of degradation of pectin. In the second step of the submerged fermentation, the production of pectinolitic enzymes were determined by quantitative test.

Quantitative tests of pectinolitic enzymes production

The quantitative test of PG production was performed by the method of the increase of the groups reducers formed after the action of the enzyme with dinitrosalicylic acid (DNS). The mixture contained 0.8 mL of acetate buffer 0.2 M, pH 5.0, containing 1% of pectin (Sigma-Aldrich 212.2 MM) and 0.2 mL of gross enzyme solution. The pre-incubation was performed at 45°C for 15 min in a bain marie. The reaction was blocked by adding 1 mL of DNS, and tubes were agitated immediately. The test tubes were placed in bain marie for 5 min at 45°C. The reducing sugar released acid (D-galacturonic) was quantified by the method of 3.5-acid dinitrosalicylic (DNS) proposed by Miller (1959). The standard curve was performed with solution of galacturonic acid Sigma-Aldrich G5269 with concentration of 0.9 to 4.2 µmol. It is defined thus: a unit of enzyme activity is the amount of enzyme capable of generating 1 µmol galacturonic acid per minute in the reaction conditions. Supernatant of the samples were analyzed by Spectrophotometer (UV visivel - Biospectro SP-220) at 540 nm, all the tests were performed in triplicate and with three replications.

Calculation of the activity and PG volumetric productivity

The standard curve of galacturonic acid Sigma-Aldrich G5269 with corresponding concentration of 0.9 to 4.2 µmol was used to define the enzyme concentration. Considering Equation 1 of the standard curve and the absorbance values (*y*) of each time of cultivation, the enzyme concentration (*x*) was obtained. In Equation 2, (x) is enzymatic concentration, (t) time of the reaction (in minutes) and ($\frac{1}{0,05}$) conversion factor (µmol to mL).

$$y = 0.2227x - 0.2181 \quad (1)$$

$$V = \frac{x}{t} \cdot \frac{1}{0.05} \quad (2)$$

Statistical analysis

All experiments were conducted using a totally randomized design. Analysis of variance (ANOVA) and Tukey test with 5% of significance was performed to evaluate statistical differences of the formation of halo of degradation pectin and the statistical differences of activity and volumetric productivity of PG in submerged fermentation. All experiments were performed in triplicate and with three replications.

RESULTS

The isolation of new filamentous fungi was obtained in three (3) cultivation system: rice (NFR), soybean (NFS) and corn (NFC) (Figure 1). The production of pectinolitic enzymes quantified in agar diffusion were observed in cultures of the isolates NFC 1, NFC 2, NFC 4, NFC 5 and NFC 6 identified as belonging to the genus *Aspergillus* sp. The isolates NFR 1, NFR 2 and NFS1 were identified as belonging to the genera *Rhizopus* and NFC 3 belonging to the genus *Cladosporium*, without enzymatic production (Figure 1). The initial experiments showed that the isolated NFC 2 obtained for substrate of corn bran obtained greater formation of halo degradation of pectin (2.3±0.27 cm) and the lowest production was obtained on the cultivation of isolated NFC 4 (1.6±0.2 cm) (Figure 1). In this step, the reference strain was used *Aspergillus parasiticus* as positive control of pectinolitic enzymes in the proposed system of study showing the formation of halo of pectin degradation. Halo degradation pectin of *A. parasiticus* was 1.0±0.2 cm (Figure 2). After quantification by agar diffusion methods, the submerged fermentation of *Aspergillus* NFC 2 in culture (SM) at 28 and 37°C was performed to evaluate the increase in PG production.

In order to assess a system of cultivation in submerged fermentation, tests were carried out by proposing cultivation in culture medium (SM) with isolated *Aspergillus* NFC 2 of greater potential for the enzyme production. Figure 2A to C presents the first step of the submerged fermentation of isolated *Aspergillus* NFC 2 in medium SM 28°C with evidence of enzymatic production observed by pectin degradation of the medium. It is possible to observe a halo of degradation of isolated *Aspergillus* NFC 2 in this condition of cultivation (Figure 2A). Cultivation of the reference fungi showed the enzymatic production by observation of pectin degradation of the medium; however, with minor intensity (Figure 2B). The negative control did not show degradation of pectin medium (Figure 2C). These results confirmed a higher potential of *Aspergillus* NFC 2 in the production of pectinolitic enzymes.

In another step of process definition, cultivations were performed with the *Aspergillus* NFC 2 at 28 and 37°C. After activation in PDA medium, an inoculum was obtained and transferred in Erlenmeyer's flasks 500 mL with 25ml of culture medium SM incubated in static system for 5 days at 28 and 37°C. One aliquot was collected at the end of the cultivation and evaluated in the agar diffusion method. Halo degradation pectin of isolates

a

New fungal strains	*Brans*	*Degradation halo formation*	*Agar diffusion essay with pectina 1% (cm)*	*Fungal identification*
NFR 1	RICE	0(3)	Wp	*Rhizopus sp* *(g-h)*
NFR 2	RICE	0(3)	Wp	*Rhizopus sp* *(g-h)*
NFS 1	SOY	0(3)	Wp	*Rhizopus sp* *(g-h)*
NFC 1	CORN	3(3)	1.5(0.04)	*Aspergillus sp* *(b-e)*
NFC 2	CORN	3(3)	2.3(0.27)	*Aspergillus sp* *(b-e)*
NFC 3	CORN	0(3)	Wp	*Cladosporium sp* *(f)*
NFC 4	CORN	3(3)	1.6(0.2)	*Aspergillus sp* *(b-e)*
NFC 5	CORN	3(3)	1.8(0.04)	*Aspergillus sp* *(b-e)*
NFC 6	CORN	3(3)	2(0.55)	*Aspergillus sp* *(b-e)*
NFC 7	CORN	0(3)	Wp	*Aspergillus sp* *(b-e)*

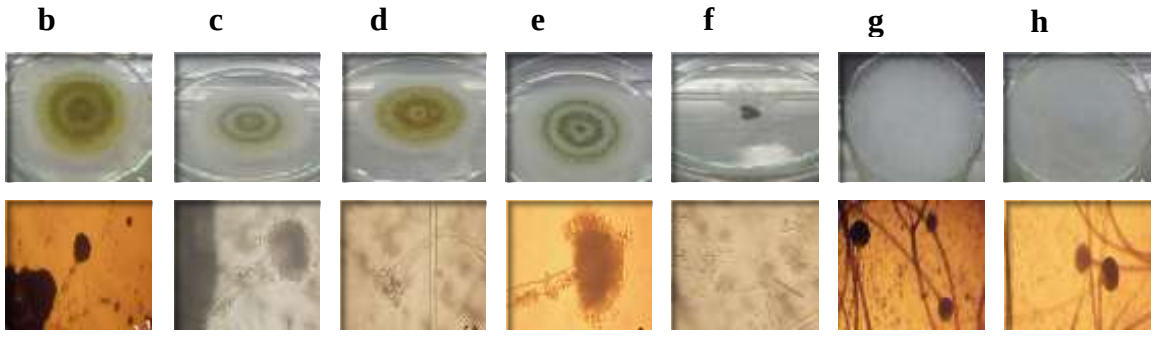

Figure 1. New fungal strains producers of pectinolytic enzymes. a- identification and characterization of fungi producers of pectinolytic enzyme. b-h- corresponding to the species by morphology and reproductive structure. Mean (standard error). NFR– new fungal strain of rice; NFS– new fungal strain of soy; NFC- new fungal strain of corn. Wp– without production. 0(3) absent in three replications and 3(3) present in three replications.

were measured (Figure 2D). The occurrence of growth was observed in both cultivation systems. However, the enzymatic production measured by degradation of pectin was greater at 28°C ($p>0.02$) (3.37cm), and lower at

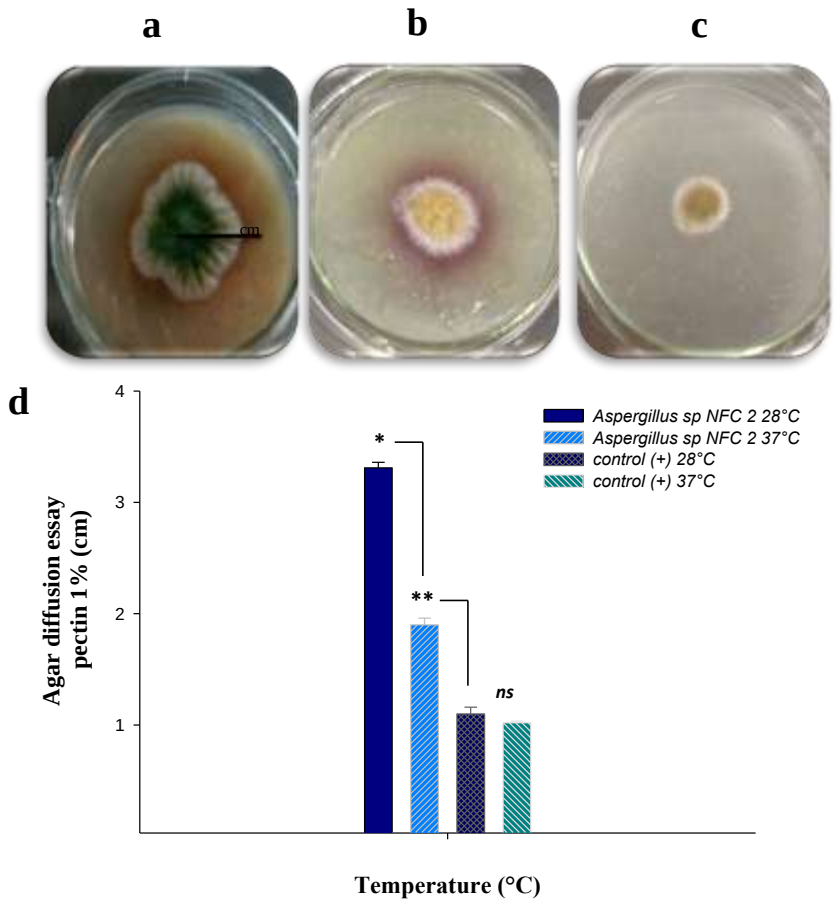

Figure 2. Analysis of the pectinolytic enzymes production in agar diffusion assay. Morphology of *Aspergillus* NFC 2 at 28°C (a). Morphology of positive control *Aspergillus parasiticus* at 28°C (b) and morphology of negative control *Aspergillus* at 28°C (c). Agar diffusion assay of *Aspergillus* NFC 2 and *Aspergillus parasiticus* (control +) in medium culture with measure of the degradation of pectin 1% at 28 and 37°C (d). *p>0.02; **p>0.007.

37°C (1.87 cm). This enzyme production in the medium SM was greater than the values of degradation of pectin obtained in previous experiments (Figure 1). The enzymatic production of *Aspergillus* NFC 2 in submerged cultivation was significantly higher (p>0.007) with reference to the positive control (1.90 cm) both at 28 and 37°C (Figure 2). In the cultivation of the reference fungi, significant differences was not observed between the temperatures evaluated. These results confirm the optimization of production of isolate NFC 2 in medium SM 28°C.

In the third step of optimization, the cultivation of *Aspergillus* NFC 2 was performed in medium SM 28°C with quantification of the activity and volumetric productivity of PG by spectrophotometric method. Figure 3 shows the activities of PG of *Aspergillus* NFC 2 in medium SM 28°C during 120 h of fermentation. The activity of PG observed during 120 h was close to 7 $U.mL^{-1}$. There was no significant difference in the activity of PG between the temperatures evaluated (Figure 3). However, volumetric productivity of PG was higher (p<0.05) in the first 24 h in both cultivation systems. At 37°C, it showed greater decay of volumetric productivity of PG along the fermentation. Statistics data are shown in Tables 1 and 2.

The identification of filamentous fungi has been performed based on analysis of their microscopic structures and morphologic aspects. Microscopic observations allow identifying characteristics of hyphae, shape, arrangement, reproductive structures, conidia and the formation of spores. The macroscopic and microscopic observation of fungi allows the definition of the genus quickly and efficient definition of cultivation system based on information of the genera of the species (Vecchia and

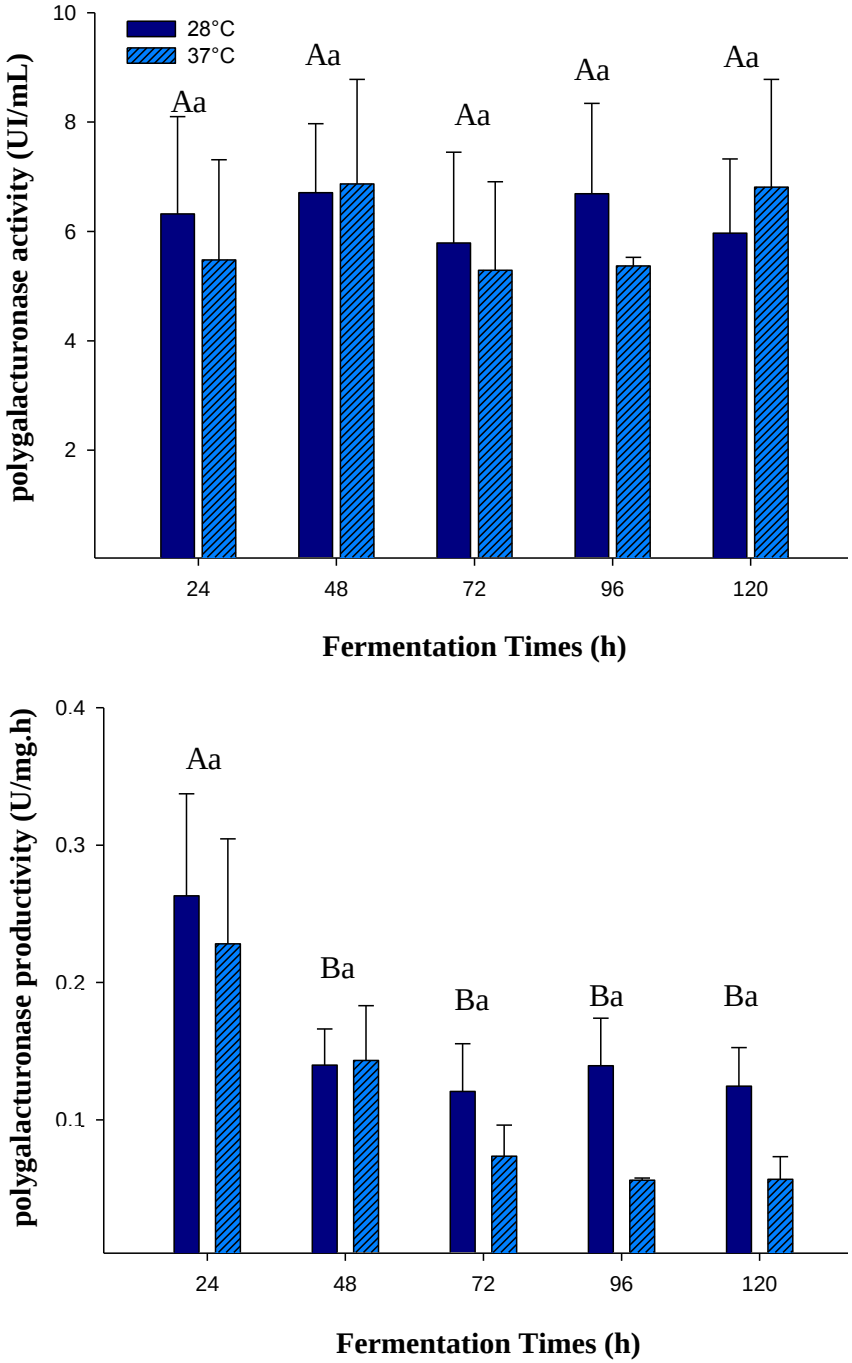

Figure 3. Enzymatic activity of the new fungal strain *Aspergillus* NFC 2. Polygalacturonase activity (a) and volumetric productivity (b) versus temperature. Means followed by the same capital letters (fermentation times) and lower case letters (temperature) do not differ significantly by the Tukey test ($p < 0.05$).

Castilhos-Fortes, 2007). Griebeler et al. (2015) made the selection of filamentous fungi producers of various compounds, including pectinase. Of their selections, 32.7% were described as microorganisms producers of pectinolytic enzymes with major halo degradation pectin to the species *Aspergillus* (2.8 cm) and *Penicillium* (3 cm) for agar medium with 1.25% of pectin. Marchi et al. (2006) analyzed activities of pectinolytic of 45 isolates

Table 1. Volumetric productivity of PG by *Aspergillus* NFC 2 in submerged fermentation conditions.

Treatment	37°C		28°C	
	Volumetric productivity	Standard error	Volumetric productivity	Standard error
T1	0.2282^{Aa}	±0.04402	0.2631^{Aa}	±0.0428
T2	0.1432^{Ba}	±0.0230	0.1399^{Ba}	±0.0151
T3	0.0736^{Ba}	±0.0130	0.1208^{Ba}	±0.0200
T4	0.0560^{Ba}	±0.0009	0.1395^{Ba}	±0.0199
T5	0.0568^{Ba}	±0.0094	0.1245^{Ba}	±0.0162

Evaluation of capital letters in the columns and lowercase letters in the lines. Means followed by the same letter are not significantly different at the level of 5% probability by the Tukey test. Legend: treatment is fermentation times.

Table 2. Activity of PG by *Aspergillus* NFC 2 in submerged fermentation conditions.

Treatment	37°C		28°C	
	PG activity	Standard error	PG activity	Standard error
T1	6.32Aa	±1.0290	5.48^{Aa}	±1.0577
T2	6.71Aa	±0.7286	6.87^{Aa}	±1.1077
T3	5.80Aa	±0.9604	5.30^{Aa}	±0.9386
T4	5.80Aa	±0.9604	5.38^{Aa}	±0.09145
T5	5.98Aa	±0.7813	6.81^{Aa}	±1.1396

Evaluation of capital letters in the columns and lowercase letters in the lines. Means followed by the same letter are not significantly different at the level of 5% probability by the Tukey test. Legend: treatment is fermentation times.

of *Alternaria solani* by agar medium diffusion evaluated by halo degradation of 1.48 cm with 3% pectin. Farias et al. (2015) also analyzed the pectinolytic enzymes production. The results showed values greater than 1.40 cm halo degradation pectin at 1.25%. In this work, higher halo degradation of (3.37 cm) with agar medium was obtained at 1% of pectin.

Agar diffusion methods have been used for analysis of detection of biomolecules production. Palumbo and O'Keeffe (2014) analyzed different species of genus *Aspergillus* employing this technique. Each isolate filamentous fungi were analyzed for the production of enzyme pectinase with employment of the agar diffusion method and the observation of halo of characteristic degradation by consumption of pectin in the medium. Zhang et al. (2015) used methods of agar diffusion for analysis up to species, identifying *Aspergillus fumigatus*. The initial analysis of the isolated fungus was performed by the agar diffusion method with the image collection and analysis of morphogenesis by optical microscopy. The identification until species was confirmed by molecular techniques with DNA sequencing where PCR primers were used. In other works, techniques for obtaining identification until species were combined. Niazi et al. (2014) employed techniques for the identification of fungi which cause aspergillosis with analyzes of macro and microscopic structures of the fungi, followed by PCR techniques, LightCycler and Elisa.

The employment of practical methods in the selection of new isolates for the production of microbial enzymes relates to the consumption of specific substrate. Robl et al. (2013) analyzed the enzymatic production of hemicelullases and related enzymes, with use of agar aesculin and agar with specific substrate evaluating the halo degradation for the initial selection. Glinka and Liao (2011) analyzed the production of pectin metilesterase (PME), polygalacturonase (PG) and pectin lyase (PL) through activity tests in the agar diffusion assays. Downie et al. (1998) also examined the quantification of pectin metilesterase activity by agar diffusion method.

The enzymatic activity and volumetric productivity of PG of *Aspergillus* NFC 2 in submerged fermentation were higher than 28°C with 24 h of fermentation. Menezes et al. (2006) studied the enzymatic activity and volumetric productivity of PG in solid fermentation using wheat bran (66.75%), humidity of 62.5% and with 64 h of fermentation of isolate of *A. niger*. The enzymatic activity and volumetric productivity obtained were of 12.01 and 0.09 $U.mL.h^{-1}$, respectively. Barman et al. (2015) evaluated the production of pectinase by *A. niger*, the

activity of PG obtained was 6.6 $U.mL^{-1}$. In this study, the volumetric productivity of PG of *Aspergillus* NCF 2 in submerged fermentation with medium SM 28°C was three (3) times more with values close to 0.3 $U.mg.h^{-1}$. The optimization of production processes is required to achieve high product yields (Brandi et al., 2014).

Zeni et al. (2011) evaluated the selection of filamentous fungi producers of PG. Out of total of 107 isolates, 15 isolates were previously identified as *A. niger*, *Penicillium* sp. and selected as potential producers of this enzyme. The production of PG obtained was greater than 3 $U.mL^{-1}$. After study of optimizing the enzymatic activity was 13 times higher than the initial values.

The enzymatic activity and volumetric productivity of PG obtained in this work characterizes the *Aspergillus* NCF 2 as a benchmark for the production of pectinolitic enzymes, therefore, their production was superior to the other remaining fungal isolates and higher than those researched in the literature. New studies of process optimization will certainly lead to higher incomes in the production of pectinolitic enzymes.

Conclusion

The tests employed in this study made it possible to obtain new fungal isolates characterizing the isolate NFC 2 with high potential for the production of pectinolitic enzymes. It allowed in a practical and efficient way, advance in the definition of cultivation systems of filamentous fungus with high productivity in submerged fermentation.

Conflict of Interests

The authors did not declare any conflict of interests.

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Abbreviation

ANOVA, Analysis of variance; **DNA,** deoxyribonucleic acid; **DNS,** dinitrosalicylic acid; **NFC,** new fungal strain of corn; **NFR,** new fungal strain of rice; **NFS,** new fungal strain of soy; **PCR,** polymerase chain reaction; **PDA,** potato dextrose agar; **PG,** polygalacturonase; pH, hydrogen potential; **PL,** pectin lyase; **PME,** pectin metilesterase; **SM,** culture medium proposed.

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Full Length Research Paper

Halophile isolation to produce halophilic protease, protease production and testing crude protease as a detergent ingredient

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Halophilic enzymes are potentially useful in many industries, particularly in food fermentation, pharmaceutical, textile, and leather for the treatment of saline and hypersaline wastewaters. In this study, a halophilic bacterium was isolated from saltpan environment, and was identified as *Bacillus* sp. Mk22 through biochemical test and 16S rRNA gene sequencing. During protease screening, the isolates produced 24 mm clear zone around the bacterial colony. The maximum production of proteases was due to the following conditions: 45°C, pH 8, 12% NaCl, carbon source glucose, nitrogen source skim milk powder and 42-h culture time, respectively. The protein was purified 16.5 fold, having 24.01% recovery, in DEAE-cellulose chromatography and 64 kDa molecular weight. Ca and Zn enhanced protease activity, while Hg strongly inhibited it. The protease was used to destain blood, ink, coffee and was active and stable under more than one extreme condition of high salt, pH, and temperature.

Key words: Saline environment, halophilic bacteria, protease, detergent

INTRODUCTION

Halophilic bacteria constitute a heterogeneous physiological group, including a variety of Gram-positive and negative bacteria, which grow optimally from 3 to 15% NaCl concentration, although they can also grow beyond this range (Ventosa et al., 1998). The isolation and characterization of novel industrially important enzyme from the halophilic bacteria with unique properties of salt, thermal, alkaline, and organic solvent stability may address the current demand for industrially stable enzymes in different processes (Souza, 2010). In recent years, halophilic microorganisms have been explored in different field of biotechnology (Mellado and Ventosa, 2003). Halophilic enzymes have highly negative surface charge with hydrated carboxyl groups protected by high salt concentration. Such is to avoid unfolding and maintain the solubility of protein (Joo and Kim, 2005). However, salt tolerant enzymes remain unexplored; halophilic enzyme-producing bacteria have been used for industries, particularly fish sauce or soy sauce in the form of mixed cultures (Oren, 2002).

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Different groups of enzymes are produced in halophilic bacteria particularly protease; these enzymes serve various industrial purposes (Mariana Delgado et al., 2012). Proteases are a single class of derivative enzymes that catalyze the cleavage of peptide bonds leading to total hydrolysis of proteins. They are physiologically significant for living organisms given their crucial applications in both physiological and commercial fields. Proteases are ubiquitous in various sources, such as microorganisms, plants and animals. Given that the plant and animal protease cannot meet the current global demands, microbial protease has thus gained increasing interest. Most commercial proteases are neutral and alkaline. The increase in the microbial proteases accounts for approximately 60% of the total enzyme sales in the world (Banik and Prakash, 2004). Proteolytic activity with potential industrial application has been characterized in *Halobacterium* spp. (Izotova et al., 1983). Halophilic proteases are less suitable for saline fermentation, because they require at least 12.5% (w/v) NaCl to express high activities (Ventosa et al., 1998). Protease comprises one of the most important groups of industrial enzymes with versatile applications, such as in cheese-making, meat tenderization, detergents, de-hairing, baking, waste management and silver recovery (Kumar et al., 2005)

The present study attempted to understand the enzymes used in washing detergent, as well as their efficiency in not only removing tough stains but also in being environmentally safe. Detergent industries need a new enzyme with novel properties that can further enhance the washing performance of enzyme-based detergents (Gupta et al., 1999). The stability of haloalkaliphilic *Bacillus* sp. (Gupta et al., 2005) and *Bacillus mojavensis* A21 (Haddar et al., 2009) has been reported, thus showing their strong potential in detergent industry. Laundry detergents are also popular in household dishwashing detergents and in both industrial and institutional cleaning detergents (Godfrey and West, 1996). This study primarily aimed to screen a novel/stable protease from halophiles for high industrial usage. Isolates were grown in culture media to obtain a crude enzyme for further characterization. The efficacy of the protease was tested as a detergent ingredient, which is one of the industrial applications of halophilic enzymes.

MATERIALS AND METHODS

Isolation of halophilic bacteria

Halophilic bacteria were isolated using halophilic agar (Hi-Media, Mumbai, India) composed of (g/l): Casein acid hydrolysate 10, yeast extract 5, trisodium citrate 3, potassium chloride 2, magnesium sulfate 25, sodium chloride 25, agar 20 and pH 7.2. Isolated bacteria were subjected to biochemical tests (Smibert and Krieg, 1994) and were used for screening the protease. High protease-producing bacterium was identified by 16S rRNA gene sequencing, and its morphology was examined using scanning electron microscopy (SEM).

Genomic DNA extraction

Genomic DNA extraction was conducted according to Moore (1995), using forward (AGA GTT TGA TCC TGG CTC AG) and reverse primers (ACG GCT ACC TTG TTA CGA CTT). The PCR was performed using primary heating step for 2 min at 95°C, followed by 30 cycles of denaturation for 20 sec at 95°C, annealing for 60 s at 55°C and extension for 2 min at 72°C then followed by a final extension for 7 min at 72°C. Purified DNA amplicons were sequenced using the ABI PRISMTM 3100 Genetic Analyzer (Applied Biosystems, U.S.A.). Moreover, the sequences obtained were deposited in the GenBank® (NCBI, U.S.A.). The sequences were assembled using Clustal W software version 1.82 (Thompson et al., 1994) available at http://www.ebi.ac.uk, and the identification was based on the pairwise alignment algorithms and phylogenetic tree.

Screening of extracellular protease

The protease growth medium contained (g L^{-1}) the following: NaCl 25, KCl 2, $MgSO_4$ 20, tri-sodium citrate 3, yeast extract 10, agar 20, pH 7.2 (Elevi et al., 2004). As a preparation, solid medium salts were separately autoclaved at 121°C for 15 min, cooled and then mixed with warm yeast extract-agar mixture to avoid precipitation of medium components.

Protease production by the selected isolates

The protease production medium was similar to the growth medium except that the yeast extract (10 gL^{-1}) was replaced by same quantity of skim milk. The inoculums contained 3.2- 5.3 x 10^5 CFU ml^{-1}. The production condition was 37°C, 200 rpm for 66 h, the cell growth and the enzyme activity were estimated at 6 h interval. Enzyme activity and biomass were measured at different temperatures, pH, carbon sources and nitrogen sources. The culture broth was centrifuged at 10,000 rpm for 20 min at 4°C and stored at -20°C until further analysis. The growth was monitored by measuring optical density at 660 nm (Shimadzu UV spectro-photometer [UV-1800], Japan).

Optimization of protease production at various parameters

Protease production was subjected to different culture conditions, temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65 and 70°C), pH (5, 6, 7, 8, 9, 10, 11 and 12), sodium chloride (1 to 24 at 3% interval), carbon sources (arabinose, glucose, maltose, raffinose, starch, sucrose and xylose), and nitrogen sources (beef extract, peptone, soybean powder, yeast extract, potassium nitrate and urea).

Purification of protease enzyme

The pre-chilled 80% acetone was gradually added to the culture supernatant with gentle stirring and was left for an hour and at 12,000 ×*g* centrifuge for 20 min, and dissolved in a minimum amount of 20 mM Tris HCl, pH 8.5 containing 50 mM NaCl and 0.5 mM $CaCl_2$ and dialyzed against the same buffer for 24 h. The

enzyme preparation was loaded on a Q-Sepharose HP column (1.6 cm × 20 cm), which had been equilibrated with the same buffer. The column was washed with equilibration buffer until no absorbance at 280 nm was detectable. The bound proteins were eluted by applying a linear gradient of 0.05 to 1 M NaCl at 1 ml/min flow rate. Active fractions (5 ml) were pooled and concentrated by ultrafiltration (Centricon, Amicon, USA) and used as the purified enzyme for further characterization. All the purification steps were performed at 4°C.

Protease activity assay

Protease activity was estimated through the modified Anson's (1938) method. The assay was performed at 37°C using 1% casein as a substrate and tyrosine in control. The substrate was prepared in 50 mM Tris-HCl buffer (pH 7.2) containing 2 M NaCl. The concentration of NaCl was set at 2 M in the assay system as casein was known to lose its original conformation at higher NaCl concentrations (Capiralla et al., 2002). Casein buffer solution (1 ml) was pre-incubated at 37°C for 5 min. The reaction was initiated by adding 1 ml of enzyme. After incubation for 10 min at 37°C, the reaction was terminated by adding 3 ml of 5% (w/v) trichloroacetic acid (TCA). For blank tubes, TCA was added prior to the enzyme. The content was centrifuged, and the absorbance of supernatant was measured at 280 nm. One unit of enzyme activity was defined as 1 µg of tyrosine released per minute. Protein was determined by Lowry et al. (1951) using bovine serum albumin as the standard. SDS-PAGE (10%) was performed to determine for molecular weight, following Laemmli et al. (1970).

Effect of different metal ions on the enzyme activity

The enzyme was pre-incubated at 37°C for 1 h in various concentrations (1, 5 and 10 mM) of different metallic salts for protease activity assay ($CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_2$, $HgCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$ and $ZnCl_2$).

Effect of surfactants and detergents activity in protease

The effect of inhibitors and surfactants on enzyme activity was carried out under standard enzyme assay conditions where the assay cocktail was supplemented with phenylmethyl sulphonylfluoride (PMSF), 10 mM; EDTA, 1 mM; cysteine, 1 mM; SDS, 0.1%; Tween-80, 0.1%; Triton X-100, 0.1%. The effect was assessed by comparing with the control (A control assay was done with enzyme solution without treatment agents and the resulting activity was considered as 100%). The laundry detergents used are the following: Ariel, Tide (Procter and Gamble Ltd.), Rin, Surf excel (Hindustan Lever Ltd.), and Henko (SPIC India Ltd.), which are widely used in India. Each (7 mg/ml) detergent was examined by incubating with 1 ml of enzymes (750 U) at 40°C, 200 rpm for 1 h, and then followed by enzymatic assay. The enzyme activity in the absence of detergent was taken as 100%. The commercial detergents solutions were initially boiled to denature any pre-existing enzymes at 100°C for 60 min.

Destaining efficiency of protease

The application of enzyme used as a detergent additive was studied on pieces of white cotton cloth (5 cm × 5 cm) stained with human blood, ink and coffee. The stained cloth pieces were taken in separate flasks, and the following sets were prepared: A1, A2 and A3: Flasks with 100 ml distilled water + cloth (stained with blood, ink, and coffee); B1, B2 and B3: Flasks with 100 ml distilled water + stained cloth + 1 ml detergent (7 mg/ml); C1, C2 and C3: Flasks with 100 ml distilled water + stained cloth + 1 ml detergent (7 mg/ml) + 2 ml of crude enzyme solution.

The above flasks were incubated at 40°C for 30 min. After incubation, the cloth pieces were taken out, rinsed with water and dried. Visual examination of various pieces exhibited the effect of enzyme in removing stains. Untreated cloth pieces stained with blood, ink and coffee were taken as control (Banerjee et al., 1999).

RESULTS

In this study, a total of 278 strains was isolated in saltpan environments, which belonged to 11 genera; *Alcaligenes* sp. -5, *Bacillus* sp. -38, *Bacillus subtilis* -27, *B. pumilis* -22, *B. lichenifomis* -22, *B. halodurans* -14, *Chromo halobacter* -8, *Clostridium* sp. -14, *Escherichia coli* -24, *Enterobacter* sp. -11, *Halomonas* sp. -8, *Halobacillus* sp. -34, *Idiomarina* sp. -20, *Klebseilla* sp. -15 and *Pseudomonas* sp. -16. The 16S rDNA gene sequences of *Bacillus* Mk22 by Ashokkumar and Mayavu (2014), consist 830 nucleotides and were submitted to GenBank (National Center for Biotechnology Information, USA) with accession numbers JF794553. The *Bacillus* Mk22 is rod-shaped, catalase positive, and its growth range was from 30 to 40°C, from pH 6 to 8.5, and from 0.5 to 12% NaCl (Figure 1). The 16S rRNA gene sequence-based phylogenetic relationships of *Bacillus* sp. Mk22 (830 nucleotides) were closely related to those of genus *Bacillus* (Figure 2).

Bacillus Mk22 secreted protease with maximum activity after 7 days; clear zones (24 mm) indicated high proteolytic activity, which was similar to previously reported data (Amoozegar et al., 2008). Protease production was tested at different parameters, in which the maximum production was attained at 45°C (Figure 3), pH 8 (Figure 4), and 12% NaCl (Figure 5). The protease activity was also significantly high in the presence of glucose (Figure 6) and skim milk (Figure 7) as compared to other substrates or effector molecules (Table 1). The purification of the bacterial protease was as shown in Table 2; the molecular weight of the protease was 64 kDa, while that of halophilic protease-producing bacteria ranges between 15.5 kDa in *Bacillus mojavensis* A21 (Haddar et al., 2009) and 69 kDa (Xin et al., 2011). In addition, Ca and Mg stimulated the protease activity, but $HgCl_2$ strongly inhibited it (Table 3).

Proteases are mainly applied in industrial production of commercial detergents to enhance their washing efficiency. Hence, detergent enzymes are expected to possess ability to withstand highly harsh conditions, such as high alkaline pH and in the presence of salts, surfactants, and other detergent ingredients. Therefore, protease stability in the presence of commercial

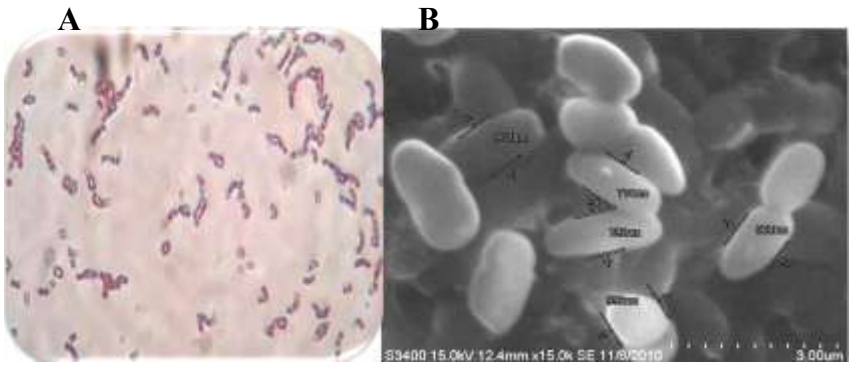

Figure 1. Morphology of *Bacillus* sp. Mk22. Microscopic (A) and scanning electron microscopic (B) view of *Bacillus* sp. Mk22 (> 31000× magnification).

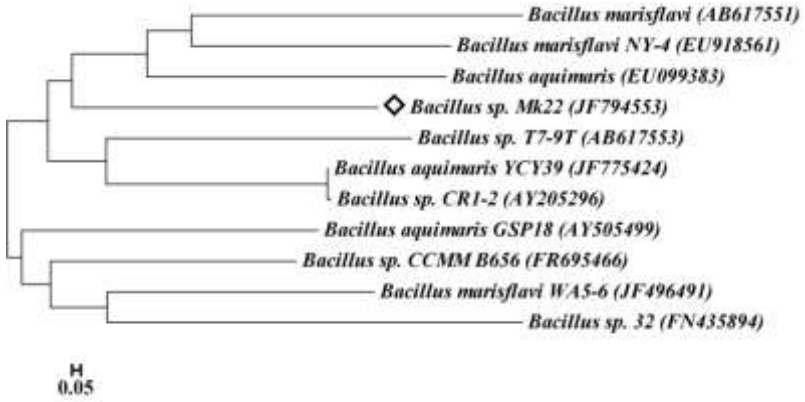

Figure 2. Phylogenetic relationship of B*acillus* sp. Mk22 and other *Bacillus* species. Genbank accession numbers are given in parentheses. Bar line 0.05 substitutions per 100 nucleotides. Tetra angle indicates the present study strain. The tree was constructed using the neighbor-joining algorithm.

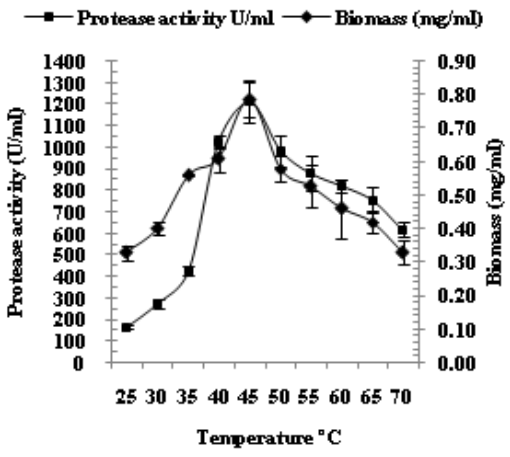

Figure 3. Protease activity and biomass at different temperatures.

detergents remains a desirable trait. The protease produced by *Bacillus* sp. Mk22 in this study significantly removed blood, ink and coffee strains at 40°C (Figure 8). The residual protease activity of *Bacillus* sp. Mk22 in the presence of various surfactants is shown in Table 4.

DISCUSSION

Halophiles have been perceived as a potential source of industrially important enzymes having exceptional stabilities. The present study intended to screen stable proteases from halophiles. In this study, protease-producing halophilic bacterium, namely, *Bacillus* sp. Mk22 was likewise isolated from saltpan and similarly halophilic bacterium *Bacillus* sp. T7-9T (AB617553) was isolated from the saltpan in South Korea (Na et al., 2011). Saltpan environment presents not only halophilic bacteria

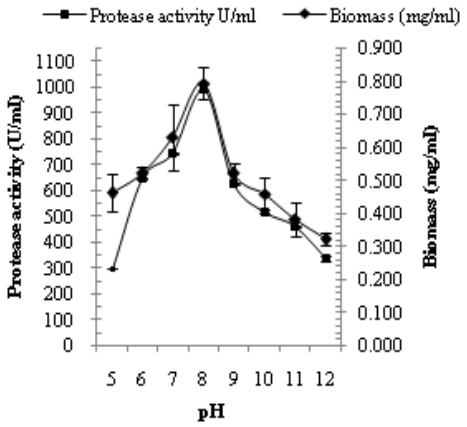

Figure 4. Protease activity and biomass at different pHs.

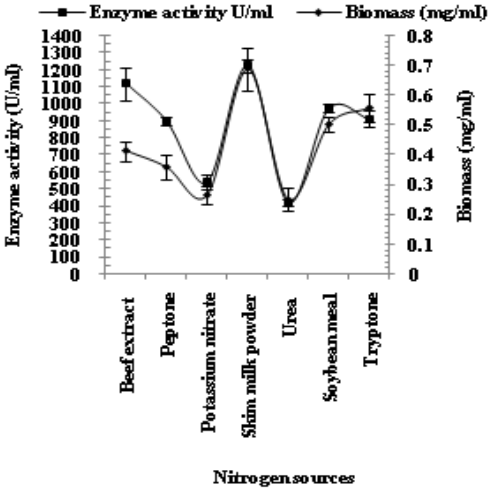

Figure 7. Protease activity and biomass with different nitrogen sources.

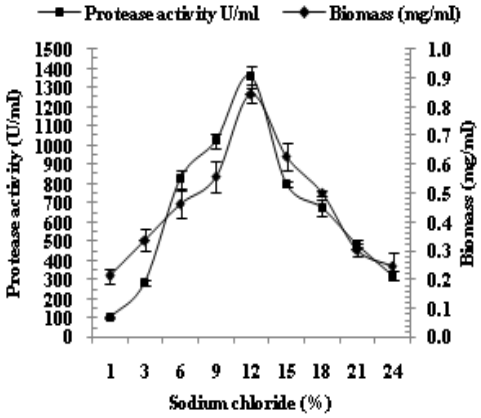

Figure 5. Protease activity and biomass at different NaCl concentrations.

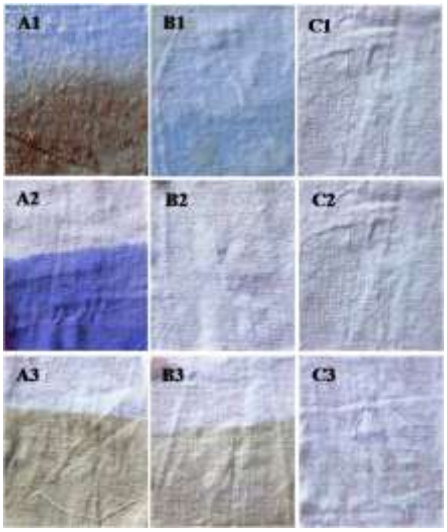

Figure 8. Supplementation of enzyme preparation in five detergents could significantly improve the cleaning performance on blood (A1, B1, C1), ink (A2, B2, C2) and coffee (A3, B3, C3).: Control (A1, A2, A3), detergents only (B1, B2, B3) and detergents + enzymes (C1, C2, C3).

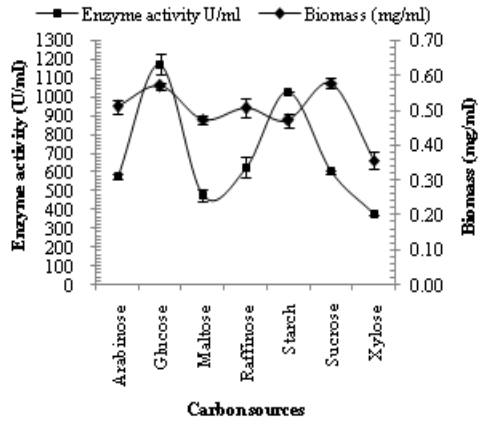

Figure 6. Protease activity and biomass with different carbon sources.

but also the non-halophilic bacteria and halophilic green microalgae, such as *Dunaliella salina* and brine shrimp *Artemia* (http://Wasterecycleinfo. com). Halophilic bacteria produce variety of enzymes particularly protease. Considerable amount of salt is used to prepare certain types of traditional fermented foods, and these salts contain halophillic bacteria producing secondary

Table 1. Conditions for maximum and minimum protease production.

Parameters	Production	Conditions	Protease activity (U/ml)
Temperature (°C)	Maximum	45	1212
	Minimum	25	165.3
pH	Maximum	8	985.7
	Minimum	5	296.3
NaCl (%)i	Maximum	12	1361
	Minimum	1	103.3
Time	Maximum	42	53
	Minimum	0	0.8
Carbon sources	Maximum	Glucose	1174
	Minimum	sucrose	371.3
Nitrogen sources	Maximum	Skim milk	1225
	Minimum	Urea	415.7

Table 2. Specific activity, recovery and purification fold of protease from *Bacillus* sp. Mk22.

Enzyme	PS	VS (ml)	TP (mg)	TA (U/ml)	SA (U/mg)	P (fold)	R (%)
Protease	CS	100	205.2	4802	23.40	0.0	100
	AMSP	25	42.60	1966	46.15	1.97	40.94
	DEAE	5	0.62	472	761.29	16.50	24.01

PS, Purification steps; CS, culture supernatant; AMSP, ammonium sulfate precipitation 80% saturation and dialysis; DEAE, DEAE cellulose chromatography; VS, volume of sample; TP, total protein; TA, total activity; SA, specific activity; P, purification; R, recovery.

Table 3. Effect of various metal ions on the activity of protease from B*acillus* sp. Mk22.

Metal	Protease activity (%)		
	1 mM	5 mM	10 mM
Control	100	100	100
$CaCl_2$	152	121	102
$CoCl_2$	93	75	96.2
$CuCl_2$	54	32	25
$FeCl_2$	104	35	26
$HgCl_2$	5	0	0
$MgCl_2$	89	107	158.3
$MnCl_2$	87	92	102
$ZnCl_2$	133	78	46.7

metabolites. Salt-rich food products are especially popular in the world, such as ‘Jeotgal’ (a traditional Korean fermented seafood), ‘fugunoko nukazuke’ (Japan’s fermented with salt puffer fish ovaries in rice bran) and ‘nam-pla’ (Thailand’s fish sauce). Although, little is still known about the microorganisms involved in

Table 4. Residual activity of protease from *Bacillus* sp. Mk22 in various surfactants.

Surfactant (concentration)	Residual activity (%)
Control	100
Phenylmethyl sulphonylfluoride (PMSF), (10 mM)	50
EDTA (1 mM)	89
Cystine (1 mM)	73
SDS (0.1%)	103
Tween-80 (0.1%)	74
Triton X-100 (0.1%)	93

the preparation of these foods, the potential of halophilic anaerobic fermentative bacteria in anaerobic treatment of saline waste waters has been reported (Kapdan and Erten, 2007).

Moderate halophilic proteases are significantly applied in biotechnology. Zavaleta and Fernandez (2007) and Moreno et al. (2007) isolated the halophilic bacteria from saltpan, and the isolated bacteria produced amylase, protease, lipase, DNase and pullulanase. Salt-tolerant proteases have high potential in the production of salt-fermented foods. However, literature on the salt-tolerant protease from halophiles, Archaebacteria and fungi remains scarce (Johnvesly and Naik, 2001). For protease production, temperature is one of the most critical parameters requiring control during the bioprocessing (Chi et al., 2007). Enzyme production is strongly influenced by temperature. The isolated bacterium *Bacillus* sp. Mk22 could tolerate high NaCl concentration and could grow on a wide range of temperature and pH. Halophilic enzyme was stable at high salt concentration and low water activity as described by Ruiz and De Castro (2007). In the present study, pH 8 produced the maximum enzyme activity, and a similar trend has been observed by various authors (Patel et al., 2006; Ganesh Kumar et al., 2008).

The 12% NaCl concentration enhanced the production, and the further increase of salt concentration significantly reduced the activity. Patel et al. (2006) have reported that maximum protease production was attained at 12% NaCl from the *Bacillus* sp. In the present study, glucose showed maximum protease production, which has been similarly reported by researchers who experimented with different type of sugars, such as lactose, maltose, sucrose, glucose and fructose (Malathi and Chakraborty, 1991). Skim milk powder produced maximum enzyme activity, while urea showed otherwise. The molecular weight of the protease investigated in this study was 64 kDa. A similar result was observed by Ravindran et al. (2011), who reported the molecular weight of protease of *B. cereus* was 66 kDa. In the present study, high protease activities were attained in Mg^{2+}, with $MnCl_2$ stimulation while $HgCl_2$ inhibited the activity. Similarly, Ca^{2+} and Mg^{2+} have been reported to increase protease activity of *B. cereus* SV1 (Manni et al., 2008). Proteases are the highest selling industrial enzymes. Their sales are projected to increase in the coming years given their applications in detergent formulations, peptide synthesis and protein processing (Chandel, 2007). In the past year, with the growing population, the demand for enzymes, particularly protease, has likewise increased.

Daily man-made and natural activities promote hypersaline environments. Therefore, halophiles and their enzymes may also be utilized in bioremediation of saline in modern day industries. The isolation and characterization of novel halophilic species that produce new enzymes may provide better opportunities. In general, majority of the commercially available enzymes are unstable in the presence of bleaching/oxidizing agents. The increased usage of these proteases as detergent additives is mainly due to the enzymes' cleaning capabilities in environmentally acceptable, non-phosphate detergents. In addition, to improve washing efficiency, the use of these enzymes allows lower water temperature and shorter activity period, often after preliminary soaking.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Microbial community structure and chemical composition from dark earth in a native archaeological site of the lower Amazon

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The microbial community structure from dark earth in a native archaeological site of the Lower Amazon was analyzed by PCR-DGGE, using 16S rRNA gene for prokaryote population and 18S rDNA and ITS regions (using clamp GC) for the eukaryote population. The bands were excised from gel and re-amplified for sequencing. The diversity found according to the region of amplification showed same profiles for the two primers pairs. The bacteria genus were: *Bacillus, Klebsiella, Pantoea, Enterobacter, Lactobacillus, Escherichia, Leuconostoc* and actinobacterias as *Streptomyces* and *Microbacterium*. Among the fungal community was *Zygosaccharomyces, Lachancea, Saccharomyces, Cladosporium, Candida, Penicillium* and Uncultured ascomycota and zygomycete were found. Molecular approaches revealed microbial groups that have never been reported in Lower Amazon soil as the *Leuconostoc mesenteroides, Lactobacillus casei* and *Lactobacillus paracasei* bacteria's and *Lachancea meyersii* yeast. The soil pH was ~6.5; the soil had high levels of minerals with exception of Na (not detected) and Al (~0.2 mg/dm^3). The organic matter was 3.5 dag/kg. This study also shows that the Amazon soil is rich in minerals. This can be an important factor in the species richness in the Amazon region. The present data show that the Lower Amazon represents a vast resource for the biotechnology area.

Key words: PCR-DGGE, fungi and yeast, soil microdiversity, soil chemical.

INTRODUCTION

The soils are biodiverse ecosystems. Microorganisms are a component of these ecosystems (Pereira et al., 2006). Microorganisms are involved in biodegradation, decomposition and mineralization, and inorganic nutrient cycling in soils.

The Amazon soil contains great unexplored ground. The Terra Preta are anthropogenic soil (Lehmann et al., 2003) which are fertilized through increasing the cation exchange capacity and the nutrient content (Lehmann et al., 2003; Kim et al., 2007).

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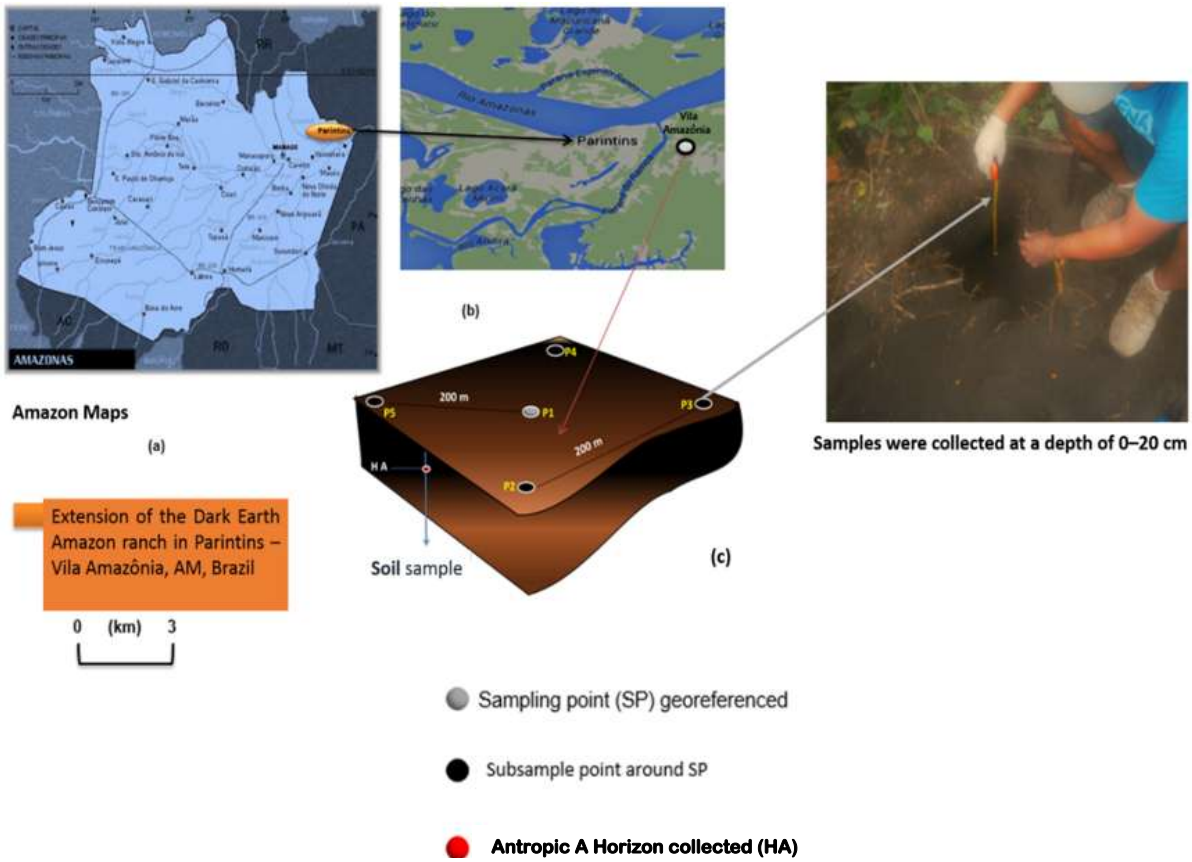


Figure 1. (a) Location of native archaeological site in Amazon, Brazil. **(b)** Site collected the samples. **(c)** Distribution of sampling point. Sampling point scheme: one composed soil sample (30 sub-samples) was collected around each sampling point.

The microbial diversity present in Amazon soils is little known. Therefore, the Brazilian government provides financial support for research. The application of molecular techniques in the microbial diversity and structure analysis of these communities has been used previously (Bossio et al., 2005; Pereira et al., 2006; Castro et al., 2008; Taketani et al., 2013; Brossi et al., 2014). The molecular approaches have been proven to be powerful tools in providing an inventory of the microbial diversity in environmental samples (Ascher et al., 2010; Silva et al., 2012).

The assessment of diversity in Amazon soil is an important aspect in the quest for maintenance of soil biodiversity (Brossi et al., 2014). A characterization of the bacterial and fungal microbial community associated with Amazon soil from dark earth in a native archaeological site of the Lower Amazon is lacking. Furthermore, it is likely that richness of these bacterial and fungal communities is affected by anthropogenic modification of environment. The objectives of this study were: Firstly, molecular survey of the bacterial and fungal communities associated with Amazon soil, based on the sequencing of different rDNA regions, and secondly to analyze the physicochemical characteristics of this soil.

MATERIALS AND METHODS

Soil sampling

Anthropogenic Dark Earth soil samples were collected from 5 points (duplicate) in a native archaeological site of the Lower Amazon, Brazil (Figure 1).

Composited samples were collected at a depth of 0 to 20 cm. The soils were stored in sterile Nasco® plastic in 4°C bags for further use.

DNA extraction and PCR-DGGE

Sample (approximately 0.25 g soil wet weight) of soil was used for DNA extraction by using a NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). Total DNA was used for PCR amplification of prokaryote and eukaryote ribosomal target regions, for PCR-DGGE analyses. Two primers sets were used for the microbial population. Primers and analysis conditions are given Table 1.

Bands from the PCR-DGGE gels were excised and were reamplified using the same primers for prokaryote and eukaryote (Table 1). The amplicons were sequenced by Macrogen Inc. (Seoul, South Korea). GenBank searches

Table 1. DGGE-PCR primers used to detect eukaryote and prokaryote community from dark earth in a native archaeological site of the Lower Amazon.

Primer	Sequence (5' – 3')	Population	Target	PCR	DGGE	References
968Fgc	AAC GCG AAG AAC CTT AC GC clamp connected to the 5' end of 968f	Bacterial	V6-V8 region of the 16S rRNA gene	Condition 1	16h at 70 V at 60°C.	[b]Magalhães et al. (2010)
1401r	CGG TGT GTA CAA GAC CC					
ITS1fGC	TCC GTA GGT GAA CCT GCG G GC clamp connected to the 5' end of ITS1gc	Fungal	ITS region of the rDNA	Condition 1	16h at 70 V at 60°C.	Wallis et al. (2010)
ITS4r	TCCTCCGCTTATTGATATGC					
338fGC	GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG GC clamp connected to the 5' end of 338fgc	Bacterial	V3 region of the 16S rRNA gene	Condition 1	6h at 70 V at 60°C.	Magalhães et al. (2010)
518r	ATT ACC GCG GCT GCT GG					
NS3fGC	GCA AGT CTG GTG CCA GCA GCC GC clamp connected to the 5' end of NS3gc	Fungal	18S region of the rDNA	Condition 2	16h at 70 V at 60°C.	Magalhães et al. (2010)
YM951r	TTG GCA AAT GCT TTC GC					

GC clamp - CGC CCG CCG CGC GCG GCG GGC GGG GCG GG , f - forward primer; r - reverse primer; Condition 1 - Denatured for 5 min at 95°C. 30 cycles: denaturing at 92°C for 60s, annealing at 55°C for 60s and extension at 72°C for 60s. Final extension for 10 min at 72°C. condition 2 - 35 cycles instead of 30.

(http://www.ncbi.nlm.nih.gov/BLAST/) were performed to determine the species of the obtained sequences. The PCR-DGGE gels were analyzed for Bio-Numerics software (version 1,5, Applied Maths, Kortrijk, Belgium) for determining the amplicons diversity.

Physico-chemical analysis of the soil

The physical and chemical characteristics of Amazon soil from dark earth in a native archaeological site of the Lower Amazon were analyzed. The soils were analyzed in duplicate according to Embrapa (1997). The followings were determined: The value of pH, concentration of hydrogen + aluminium (H + Al), calculation of exchangeable bases (SB), zinc (Zn), iron (Fe), manganese (Mn), copper (Cu), nickel (Ni), arsenic (As), potassium (K), phosphorus (P), aluminum (Al), magnesium (Mg), organic matter (OM), cadmium (Cd), lead (Pb), sodium (Na) and mercury (Hg). For statistical analysis, the SAS System 9.1 (SAS Institute Inc., Cary, NC, USA) was used.

RESULTS

Use of different primers to assess eukaryote and prokaryote communities from dark earth in a native archaeological site of the Lower Amazon

The bacterial and fungal DGGE profiles of Amazon dark earth in a native archaeological site of the Lower Amazon are shown in Figure 2. These profiles exhibited the species abundance. For eukaryote, primer pair ITS1fGC/ITS4r and NS3fGC/YM951r were able to provide a diversity of bands and to differentiate filamentous fungi (*Penicillium* and *Cladosporium)* and yeasts (*Saccharomyces, Zygosaccharomyces, Candida* and *Lachancea).*

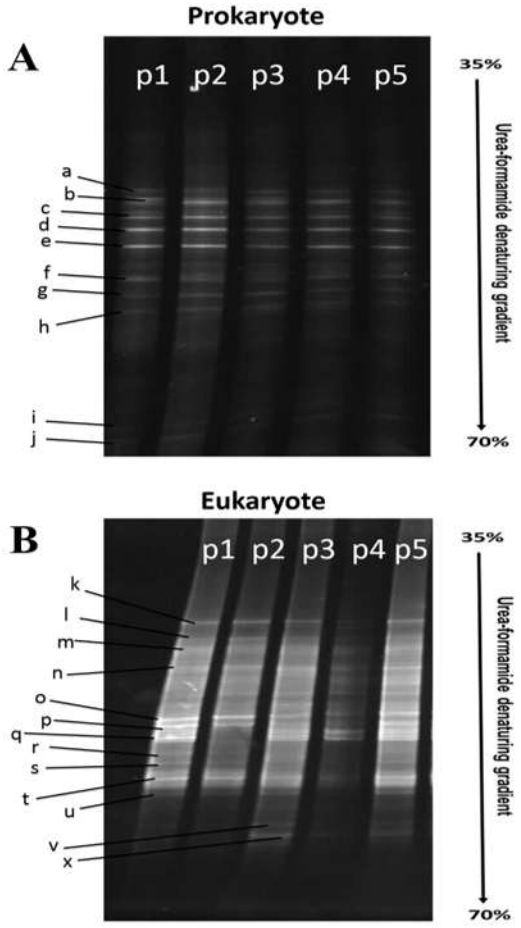
For eukaryote, the primers 338fGC/518r and 968fGC/1401r were able to demonstrate variety of bands with the appearance of *Bacillus, Klebsiella, Enterobacter, Pantoea, Escherichia, Lactobacillus, Leuconostoc* and actinobacterias.

The obtained results using different pairs of primers, show a diverse PCR-DGGE profile, suggesting the presence microbial consortium. This has high relevance in terms of the efficiency of the characterization of microbial diversity of Amazon dark earth in a native archaeological site of the Lower Amazon.

Identification of microbial communities from dark earth in a native archaeological site of the Lower Amazon

Table 2 and Figure 2 showed the molecular diversity of bacterial and fungal from dark earth in a native archaeological site of the Lower Amazon. Identical profiles were obtained for the following primers pairs: Prokaryote (968fGC/1401r and 338fGC/518r) and eukaryote (ITS1fGC/ITS4 and NS3fGC/YM951r). The sequencing bands exhibited equal and higher than 98% identity with sequences available in the Gen-Bank.

In relation to the prokaryotic community analysis, the bands a to j (Figure 2a) were identified as different bacteria species. The band a was identified as *Bacillus macerans* (AB281478), band b - *Klebsiella pneumonia*



Identical profiles were obtained for the following primers pairs:
Prokaryote (968fGC/1401r and 338fGC/518r)
Eukaryote (ITS1fGC/ITS4 and NS3fGC/YM951r)

Figure 2. PCR-DGGE profiles of the Prokaryote (A) and Eukaryote communities (B) in rDNA fragments amplified from dark earth in a native archaeological site of the Lower Amazon.(A) The closest relatives of the fragments sequenced, based in search of GenBank (≥99% similarity), werebands: A - *Bacillus macerans* (AB281478), b - *Klebsiella pneumonia* (CP000964), c –*Pantoea agglomerans,* d - *Enterobacter cowanii* (FJ357832) , e - *Lactobacillus casei* (EU626005.1), f -*Escherichia coli* (EU026432), g – *Leuconostoc citreum* (FJ378896.1), h - *Streptomyces gelaticus* (EU741111.1),i - *Microbacteriumazadirachtae*(EU912487.1), j -*Lactobacillus paracasei* (AB368902.1). (B) The closest relatives of the fragments sequenced, based on a search of GenBank (≥98% similarity), were bands: k -*Zygosaccharomyces* sp. (AF017728.1), l –*Lachancea meyersii* (AY645661.1), m -Uncultured Ascomycota (GQ404775), n -Uncultured zygomycete (AY969178), o - *Saccharomyces cerevisiae* (EU019225.1), p - *Cladosporium oxysporum* (AJ300332.1), q - *Cladosporium* sp. (FJ950740), r – *Candida glabatra* (AY939793.1),s – *Candida tropicalis* (EF194842.1),t – *Candida orthopsilosis* (FN812686.1), u –*Candida* sp. (G1190714325), v - *Penicillium oxalicum* (JF309107), x - *Candida labiduridaru* (FJ623629.1). Abbreviations: p = collected points.

(CP000964), band c - *Pantoea agglomerans* (FJ388592.4),, band d -*Enterobacter cowanii* (FJ357832), band e - *Lactobacillus casei* (EU626005.1), band f - *Escherichia coli* (EU026432), band g - *Leuconostoc citreum* (FJ378896.1), band h - *Streptomyces gelaticus* (EU741111.1) ,band i - *Microbacterium azadirachtae* (EU912487.1) and band j as *Lactobacillus paracasei* (AB368902.1).

In relation to the eukaryotic community analysis (Figure 2b), the band k was identified as *Zygosaccharomyces* sp. (AF017728.1), band l - *Lachancea meyersii* (AY645661.1), band m -Uncultured Ascomycota (GQ404775), band n - Uncultured zygomycete (AY969178), band o - *Saccharomyces cerevisiae* (EU019225.1), band p - *Cladosporium oxysporum* (AJ300332.1), band q - *Cladosporium* sp. (FJ950740), band r - *Candida glabatra* (AY939793.1), band s - *Candida tropicalis* (EF194842.1), band t - *Candida orthopsilosis* (FN812686.1),band u – *Candida* sp. (G1190714325), band v - *Penicillium oxalicum* (JF309107), and band x as *Candida labiduridaru* (FJ623629.1).

C. labiduridaru (FJ623629.1) yeast and *P. oxalicum* (JF309107) filamentous fungi were found in only samples of the points 3, 4 and 5 numbers. Figure 3 describes the microbial genus abundance in dark earth in a native archaeological site of the Lower Amazon. The bacterial genus of greater abundance was *Lactobacillus* and the fungal genus of greater abundance was *Candida*.

Physico-chemical properties from dark earth in a native archaeological site of the Lower Amazon

Chemical and biochemical properties of dark earth in a native archaeological site of the Lower Amazon are given in Table 3. The soil pH value was close to neutral (~6.5). The soil had high levels of minerals with exception of Na and Al (Table 3). The organic matter was found as 3.5 dag/kg.

DISCUSSION

Studies of microbiota and soil chemistry show that the fertility of anthropogenic soils results from combination of mineral and organic components (Navarrete et al., 2010; Taketani et al., 2013). The microbial population profiles can be dependent on the PCR primers used (Anderson and Cairney, 2004; Schwarzenbach et al., 2007). When Amazon soil samples were subjected to analysis by PCR-DGGE, important information became available. Species present in low concentrations can be detected by PCR-DGGE (Pereira et al., 2011).

The results show that the eukaryote population in the Amazon soils reveals a microbial diversity, *Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp., *Zygosaccharomyces* sp. *Lachancea* sp., *Saccharomyces* sp., *Cladosporium* sp., and *Candida* sp. The large

Table 2. Molecular diversity of bacteria and fungal from dark earth in a native archaeological site of the Lower Amazon.

Point	Bacteria	Yeasty	Filamentousfungi
1	*B. macerans* (AB281478), *K. pneumonia* (CP000964), *P. agglomerans* (FJ388592.4), *E. cowanii* (FJ357832) , *Lactobacillus casei* (EU626005.1), *E. coli* (EU026432), *L. Citreum* (FJ378896.1), *S. gelaticus* (EU741111.1), *M. Azadirachtae* (EU912487.1), *L. paracasei* (AB368902.1)	*Zygosaccharomyces* sp. (AF017728.1), *L. meyersii* (AY645661.1),Uncultured Ascomycota (GQ404775), Uncultured zygomycete (AY969178), *S. cerevisiae* (EU019225.1), *C. glabatra* (AY939793.1), *C. tropicalis* (EF194842.1), *C. orthopsilosis* (FN812686.1), *Candida* sp. (G1190714325)	*C. oxysporum* (AJ300332.1), *Cladosporium* sp.(FJ950740)
2	*	*	*
3	*	**Candida labiduridaru* (FJ623629.1)	**Penicillium oxalicum* (JF309107)
4	*	**C. labiduridaru* (FJ623629.1)	**P. oxalicum* (JF309107)
5	*	**C.labiduridaru* (FJ623629.1)	**P. oxalicum* (JF309107)

*Species same of the Point 1.

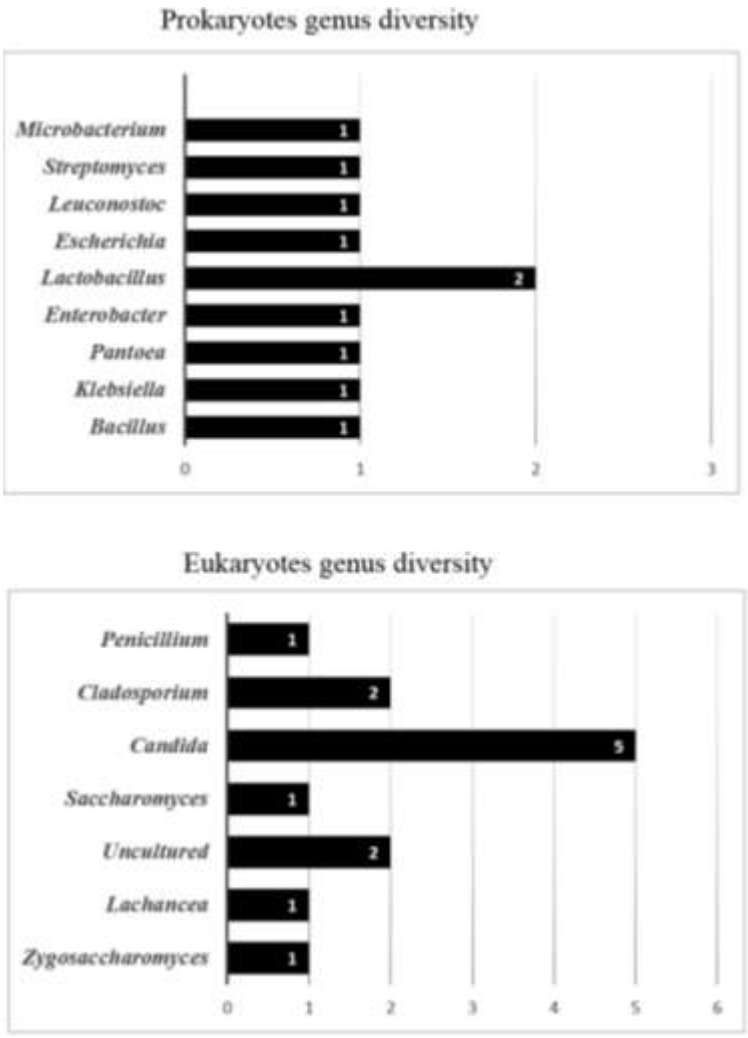


Figure 3. Microbial genus abundance from dark earth in a native archaeological site of the Lower Amazon (Numbers mean species abundance).

Table 3. Chemical and physical characteristics from dark earth in a native archaeological site of the Lower Amazon.

Point	pH	P mg/dm^3	K mg/dm^3	Mg mg/dm^3	Al mg/dm^3	Zn mg/dm^3	Fe mg/dm^3	Mn mg/dm^3	Cu mg/Kg	Cr mg/Kg
1	6.3±0.1^a	51.2±0.1^a	25±1^a	2.1±0.0^a	0.1±0.1^a	6.4±0.1^a	23.4±0.1^a	24.3±0.1^a	16.3±0.1^a	12.9±0.1^a
2	6.4±0.1^a	51.3±0.1^a	26±2^a	2.1±0.0^a	0.2±0.1^a	6.5±0.1^a	23.0±0.1^a	24.4±0.1^a	16.4±0.1^a	12.7±0.1^a
3	6.5±0.1^a	51.2±0.1^a	25±1^a	2.2±0.0^a	0.2±0.1^a	6.6±0.1^a	23.1±0.1^a	24.3±0.1^a	16.3±0.1^a	12.6±0.1^a
4	6.5±0.1^a	51.1±0.1^a	24±1^a	2.1±0.0^a	0.1±0.1^a	6.6±0.1^a	23.5±0.1^a	24.5±0.1^a	16.5±0.1^a	12.8±0.1^a
5	6.4±0.1^a	51.2±0.1^a	25±1^a	2.1±0.0^a	0.1±0.1^a	6.7±0.1^a	23.4±0.1^a	24.2±0.1^a	16.2±0.1^a	12.8±0.1^a
	Ni mg/kg	**As mg/Kg**	**Cd mg/Kg**	**Pb mg/Kg**	**Hg mg/Kg**	**Ca Cmol/dm^3**	**H+Al Cmol/dm^3**	**OM dag/Kg**	**SB mg/dm^3**	**Na mg/dm^3**
1	3.2±0.1^a	1.9±0.1^a	0.14±0.1^a	8.8±0.1^a	1.02±0.1^a	7.1±0.1^a	4.04±0.1^a	3.2±0.1^a	9.16±0.1^a	n.d
2	3.4±0.1^a	1.9±0.1^a	0.14±0.2^a	8.7±0.1^a	1.04±0.1^a	7.2±0.1^a	4.04±0.1^a	3.4±0.1^a	9.14±0.2^a	n.d
3	3.5±0.1^a	1.6±0.1^a	0.14±0.1^a	8.5±0.1^a	1.02±0.1^a	7.1±0.1^a	4.05±0.1^a	3.5±0.1^a	9.16±0.1^a	n.d
4	3.5±0.1^a	1.6±0.1^a	0.14±0.1^a	8.5±0.1^a	1.02±0.1^a	7.3±0.1^a	4.05±0.1^a	3.5±0.1^a	9.16±0.1^a	n.d
5	3.4±0.1^a	1.7±0.1^a	0.14±0.1^a	8.6±0.1^a	1.04±0.1^a	7.1±0.1^a	4.04±0.1^a	3.4±0.1^a	9.14±0.1^a	n.d

Data are average values of duplicate ± standard deviation. Different letters indicate significant differences ($P < 0.05$). K, Potassium; P, phosphorus; Al, aluminum; Mg, magnesium; OM, organic matter; pH; H + Al, hydrogen + aluminium; Zn, zinc; Fe, iron; Mn, manganese; Cu, copper; Ni, nickel; As, arsenic; Cd, cadmium; Pb, lead; Na, sodium; Hg, mercury; (SB, exchangeable bases; n.d. not detected.

variability is due the incorporating of different types and quantities of organic matter (Bissett et al., 2013). Recently, Bresolin et al. (2010) observed microbiota present in Brazilian Cerrado soil using DNA analysis by PCR-DGGE. The microbial species isolated were related to Uncultured soil fungus and Uncultured soil bacteria. Schwarzenbach et al. (2007) observed the presence of Ascomycota yeasts in sandy loam soil sample situated in Central Switzerland.

Among the genus and species of yeast and fungi found in this study; *Lachancae meyersii* is the first species in the collected genus from Amazon soil. According to Fell et al. (2004), all of the isolations of the other species of *Lachancea* have been from plants, plant products or plant-associated insects, fruits or food. The specific ecological niche of *L. meyersii* has not been determined. *Zygosaccharomyces* is a yeast genus as synonymous with spoilage. *Zygosaccharomyces* includes osmotolerant (Thomas and Davenport, 1985). These characteristics of resistant microorganisms to different environments are related to presence in soils. The *Cladosporium* genus constitutes one of the largest genus of Hyphomycetes (Mukherjee and Mittal, 2005). *Cladosporium* grows when there is not enough ventilation; sometimes on walls and wallpaper in rooms (Mukherjee and Mittal, 2005). However, recent data suggest that they are present in soil samples (Paul et al., 2008). The *Candida* genus is commonly found in soils in forest soil in Taiwan including the new species *Candida jianshihensis, Candida yuanshanicus, Candida dajiaensis* and *Candida sanyiensis* (Meyer et al., 1998). Shin et al. (2001) also found Candida genus in Korean soils.

The obtained results showed the presence of several bacterial species in Amazon soil (Figures 2 and 3 and Table 2). The bacterial species *Bacillus* sp. commonly are found in soil samples (Quirino et al., 2009).

Klebsiella, Enterobacter, Pantoea, and *Escherichia* genera were found in this study. These genera are found naturally in soil, water, and plants (Quirino et al., 2009). *Leuconostoc* and *Lactobacillus* genera are not commonly found in soil, however it was found in this study. The presence of this specie in soil is associated with plants and fruits present in vegetation Amazon.

This study also showed the presence of actinobacterias as *Streptomyces gelaticus* (EU741111.1) and *Microbacterium azadirachtae* (EU912487.1). Actinobacteria are genus colonizers in soils. Many species produce enzymes for degradation of cellulose, chitin and, in part, starch. Actinobacteria often occur in degraded organic materials (Schäfer et al., 2010). In the present work, the Streptomyces genus was also found. The Streptomyces genus is focus of research because of the produced substances and has been modified with advances in molecular biology (Souza et al., 2008). *Microbacterium* genus can be isolated from air, soil and water. Many *Microbacterium* spp. play a significant role in oil, lactone and xylan degradation, production of bio-surfactants, and as a growth promoter in plants (Lin et al., 2012).

The soil physico-chemical properties are important and how these properties could be related to microbial profiles in different soils must be evaluated (Peixoto et al., 2010). The properties affect the native microbial populations (Bresolin et al., 2010). This study showed small amounts of aluminum in the Amazon soil (Table 3). High quantities of aluminum in the soil promote the soil impoverishment (Ruggiero et al., 2002). This study also showed the Lower Amazon soil is rich in minerals. This can be an important factor in the species richness in the Amazon region.

Conclusion

In summary, this research has furthered our knowledge about microbial community structure and chemical composition of archaeological site of the Lower Amazon. The application of PCR-DGGE technique based approaches for prokaryote and eukaryote population analysis has confirmed that microbial ecosystems of Amazon soil support a wide diversity of microorganisms that may be responsible for some characteristics these soils. On the other hand, molecular approaches revealed microbial groups that had never been reported in native archaeological site of the Lower Amazon as the *Leuconostoc mesenteroides, Lactobacillus casei* and *Lactobacillus paracasei* bacteria's and *L. meyersii* yeast. The data presented adds important information that will help future studies in these environments.

Conflict of interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Abbreviations: ITS, Internal transcribed spacer, **PCR-DGGE,** polymerase chain reaction -denaturing gradient gel electrophoresis.

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