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

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Full Length Research Paper

Synergistic interaction of extracts of garlic (*Allium sativum*) and propolis against methicillin-resistant *Staphylococcus aureus*

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a public health problem, being a cause of severe diseases in hospitals and communities in general. To confront this contingency at present, the effectiveness, in combination with diverse natural products is being studied in order to inhibit this microorganism. The objective of the present work was to evaluate the combined inhibitory effects of ethanolic extracts of garlic (*Allium sativum*) and propolis (Propolis –ppl-) against MRSA strains. We tested two types of extracts: at 20 and 30% for each. Microbial resistance assays were evaluated by the macrodilution method and the combinations were assessed by isobolographic studies. The study strains were divided into two groups based on their resistance to garlic as sensitive (36.8 ± 7.4 mg/mL) and resistant (67.2 ± 8.9 mg/mL). The results show that for strains catalogued as sensitive, the combinations in both concentrations suggest a synergistic effect; on the other hand, for strains catalogued as resistant, the combinations no longer presented a synergic effect.

Key words: Synergism, isobolographic analysis, methicillin-resistant *Staphylococcus aureus* (MRSA), garlic, propolis.

INTRODUCTION

Staphylococcus aureus is a medically important microorganism. For several years, it has been recognized as the main pathogenic agent of its genus for infections of community as well as hospital origin. *S. aureus* forms parts of the Micrococcaceae family, genus *Staphylococcus*, which comprises more than 30 different species, many of

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which are natural inhabitants of human skin and mucous membranes. (Bustos et al., 2006; Camarena and Sánchez, 1997). It is a versatile pathogen that can cause diverse affectations in humans, among which the following are included: superficial lesions, such as skin abscesses and wound infections; systemic infections such as bacteremia or toxemic syndromes such as food poisoning (Espinoza and Gómez, 2007). In addition to its participation in multiple infectious processes, the staphylococci possess great clinical importance due to the progressive increase of mutations that have conferred resistance to multiple antibiotics (Jarraud et al., 2002; Gil, 2000).

The *S. aureus* strains that present resistance to beta-lactams have been denominated methicillin-resistant or MRSA (Hernández et al., 2003). In Mexico, the Hospital Network of Epidemiological Surveillance (RHOVE in spanish) has notified that the percentage of mortality among patients infected with *S. aureus* ranges between 5 and 70% and that percentages of attributable mortality can be high (up to 50%). With data derived from general, pediatric, university, and specialty hospitals, the RHOVE Network has reported that during a period of 1997-2003, S*. aureus* occupied third place in morbility and fourth in mortality (Velazquez, 2005). The great majority of MRSA are not only resistant to beta-lactams, but also to multiple antibiotics, including vancomycin, oxacillin, erythromycin, rifamycin and ciprofloxacin (Camarena and Sánchez, 1997; Filimon et al., 2009)

Due to the great problem caused by MRSA strains in humans, diverse and varied components and substances of plant origin are studied in order to inhibit and control the growth of the microorganism; thus, *Allium sativum* (garlic), a monocotyledon species belonging to the Liliacea family of Asiatic origin, whose medicinal properties have been known since ancient times, has been studied for its anti-microbial effects, which are attributed mainly to the allicin, which possesses potent inhibitory effects on certain enzymes, such as the cysteine proteinases and the alcohol-dehydrogenases, which play important roles in fungal, bacterial and virus infections (Tejerina, 2001; Bptosta, 2005; Duran et al., 2010)

Another natural substance that has also been extensively studied is propolis. Propolis (ppl) is defined as a combination of diverse substances that contain balsamic, ether oils, pollen, vitamins, among others, and that confer antifungal, antibacterial and anti-inflammatory properties, elaborated by bees (*Apis mellifera*) for protection of the beehive (Bracho et al., 2009; Marcen, 2002; Boyanova et al., 2003). Although it has been reported that the antibiotic effects vary by region, the ppl gathering season, and the solvent utilized for extraction, ppl is already being currently taken as a potent anti-microbial, especially against *S. aureus*, whose mechanisms of action include cell-wall debilitation, inhibition of protein synthesis, and inhibition of the process of replication and genetic expression (Palomino et al., 2009; Bustos et al., 2006)

The combination of antimicrobials has demonstrated to be a potent form of combating multiresistant strains. The most common way is to combine diverse pharmaceuticals; however, the combination of natural substances has shown to be a field that should be considered with greater detail, due to the good effects that it has exhibited. One of the ways that the interaction among pharmaceuticals can be evaluated is by means of isobolographic analysis. If two drugs are administered together, their effects can be as follows: (a) additive: corresponding to the simple sum of the effects that each of them produces separately; (b) sub-additive: also termed antagonists, which corresponds to a lesser effect than the simple sum of each agent separately, and (c) synergic or supra-additive: a greater effect than the sum of the effects separately of each drug. It is necessary to conduct a qualitative assay to distinguish these cases when they are solely due to the action of simple addition (Vazquez, 2005; Tallarida, 2002)

Although successful combinations of ppl with other compounds have been reported (Lozina et al., 2008; Fernandes et al., 2005), there are no antecedents of the garlic-ppl combination. The aim of present work was to evaluate the synergic effect on combining extracts of ppl and garlic *in vitro*, against MRSA strains for isobolographic studies.

MATERIALS AND METHODS

Reagents and solutions

Mueller-Hinton Broth (MHB) Becton Dickinson BBCTM Lote0340475, Mueller-Hinton Agar (MHA) BD Bioxon Lot 0006404 and Oxacillin discs (Becton, Dickinson and Co., BDBBL Sensi-disc of 1 µg Ref. 231319) were used. For propolis and garlic extracts, we used absolute ethanol (99.304%) CTR Scientific lot 03T14165 (CTR 01160) and, as reference strain, we employed *S. aureus* ATCC 29213 based on recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2010).

Formulation of the extracts

Propolis was collected from the Canatleca apiculture region (Canatlán, Durango state, Mexico); it was cleaned manually from waxes, and from the remains of plants or insects and was macerated. As for the garlic, this was acquired in the market of the locality, it was peeled and cut into short pieces of about 1 mm in diameter. The garlic plant was identified at National Polytechnical Institute-Dgo. Ethanolic extracts at 20 and 30% was prepared as follows: we weighed 20 g of propolis and 20 g of garlic, each separately, and we added 100 mL of ethanol; we allowed these to rest for a period of 8 days with occasional shaking, protected from light with a covering of aluminum foil at room temperature. In this manner, the two extracts were obtained at 20% (propolis PPL20 and garlic 20). In a similar fashion, the extract was prepared at 30% (PPL30 and garlic 30), weighing 30 g of each separately. At the end of the rest period, the extracts were filtered through Whatman filter paper and were aliquoted in 50-mL corning conical tubes. The

extracts were maintained under protection from light and under refrigeration (at 5-7°C) until the time of the study (Bptosta, 2005; Duran et al., 2010; Tallarida, 2002; Fernandes et al., 2005).

Gathering and characterization of strains

25 strains of *S. aureus* were randomly collected in healthy volunteers whose ages ranged from 1 month to 59 years. The strains were characterized as *S. aureus* by biochemical tests according to that established by McFaddin (2003) (coagulase, catalase and mannitol). In order to identify MRSA strains, we employed oxacillin discs according to guidelines established by the CLSI. ATCC29213 strain was utilized as quality control for such identification. The MRSA strains identified were maintained at -20°C in skim-milk medium and were employed later in the present study. The strains were codified X1, X2, X3, etc. A total of 25 strains were collected, and, only SARM was included in the study: X1, X8, X18, X23 and X24.

Inoculum preparation

Each strain was thawed at room temperature and later cultured on plates with MHA by means of crossed striae in four fields and incubated at 37°C for 24 h. Later, two colonies were removed and suspended in 150 mL of MHB, obtaining an initial population of between 1 × 10E5 and 1 × 10E6 colony-forming units (CFU), which constituted the inoculum, according to CLSI recommendations. Additionally, an aliquot of this inoculum was cultured on a plate with the purpose of counting the population of the inoculum expressed as CFU × 10E6. This population was selected as the basis for calculating effective concentrations 50 (EC50), as described later.

Inhibition studies

The studies were carried out in triplicate by the macrodilution method, according to that described by Taroco et al. (2010). Based on previous assays, we established the following work ranges: 25; 12.5; 6; 3; 1.5; 0.7, and 0.3% of extract v/v for garlic 20% and PPL 20%. Likewise, we utilized 25, 12.5, 6, 3, 1.5, 0.7 and 0.3% of extract v/v for garlic 30% and PPL 30%. After the corresponding inoculation, the extracts wad allowed to act for a period of 20 min and then 10 µL of each concentration (and of each series) was seeded on plates with MHA. The plates as well as the tubes were incubated at 37°C for 18-20 h. To discard the possible inhibitory effect of the ethanol used in the extracts, each strain was cultured in the presence of pure alcohol at the same dilutions utilized in the extracts, employing water as diluent.

MIC, MBC and EC50

Minimal inhibitory concentration (MIC) was taken as the concentration in which appreciable turbidity due to growth was not observed. To determine MIC, we measured absorbance by spectrometry at a wavelength of 625 nm. The plates were expressed as the population of colony-forming units (CFU) × 10E6/mL. As minimal bactericidal concentration (MBC), the concentration that did not exhibit growth on the plate was established. Effective concentration 50 (EC50) was calculated in the following manner: a) in each case, mortality was determined as the difference between the initial population (the inoculum) and the population counted on the plate of each antibiotic concentration; b) after, the percentage of effect was determined as a relationship between the average mortality of the three runs in each concentration and the initial population; c) the log was included in a graph of concentrations vs. percentage of effect, obtaining a straight line, and determining the corresponding equation; d) finally, with this equation, the concentration from which 50% of effect was calculated, and EC50 was obtained. The effects of the four groups were compared: garlic 20, garlic 30, propolis 20 and propolis 30.

Statistics and software

The normal distribution of data was calculated by means of the Shapiro-Wilks test. To verify intergroup differences, an analysis of variance (ANOVA, $p \leq 0.05$) was applied. Microsoft Excel and SPSS 19.0 softwares were employed.

Combinations

In order to evaluate the combinations between the extracts of propolis and garlic, we followed the method described by Talladira (2002) for isobolographic studies. This method has demonstrated to be a simple and objective form for evaluating combinations of pharmaceuticals and presents great statistical robustness. The additive line was established based on the average EC50 of the strains studied, as described by the methodology. The strains were grouped based on their resistance to garlic as follows: Group 1 comprised of five strains that presented an average sensitivity to the garlic extracts of 36.8 ± 7.4 mg/mL, and Group 2, made up of three strains that presented an average resistance of 67.2 ± 8.9 mg/mL. It is necessary to clarify that the groups are independent one one form the other and that these are not compared among themselves. Following the methodology, the combinations with ppl are detailed in Tables 1 and 2 for groups 1 and 2, respectively. After making the cultures in broth and seeds which were plated, the EC50 was calculated as has been previously described. The Student t test was applied for comparing average theoretical concentrations (EC50t) against experimental concentrations (EC50e), as indicated in Talladira's methodology. The experimental proportions of each antimicrobial were determined in order to outline the corresponding isobologram as described previously in the methodology and finally, the interactions index was determined.

RESULTS

Nine of the total strains (36%) from healthy carriers were MRSA. For the garlic extract, minimal inhibitory concentrations (MIC) were 46.13 ± 18.51 mg/mL and 67.79 ± 26.7 mg/mL for extracts at 20 and 30%, respectively. With regard to the propolis, MIC were 18.9 ± 6.4 mg/mL and 14.1 ± 10.2 mg/mL for the extracts at 20 and 30%, respectively.

The result obtained in the isobolographic study of group 1 strains for extracts at 20% as well as at 30%, respectively, of garlic and propolis employed for the inhibition of *S. aureus* are depicted in Table 3.

According to the study for Group 1 strains treated with extracts at 20 and at 30%, respectively, a statistically significant difference was found in the inhibitory concentration of antibiotics in the experimental stage (EC50e) in reference to the theoretical concentration (EC50t) against the microorganism ($p < 0.05$).

The proportionality of the concentrations in the Group 1 combinations are depicted in Table 4 and the isobologram in Figure 1.

Table 1. Combinations used in the isobolographic study for extracts at 20% and 30% propolis and garlic for inhibition of Group 1 MRSA strains.

Group 1 strains					
Extracts at 20%			Extracts at 30%		
EC50 Garlic (mg/mL)	EC50 Propolis (mg/mL)	Zt (mg/mL)	EC50 Garlic (mg/mL)	EC50 Propolis (mg/mL)	Zt (mg/mL)
18.4			17.3		
9.2	0.26	9.46	8.5	0.165	8.67
4.6	0.13	4.73	4.2	0.08	4.28
2.3	0.06	2.36	2.1	0.04	2.14
	0.53			0.33	

EC50: Effective concentration, 50; Zt: Sum of the fractions of both extracts.

Table 2. Combinations used in the isobolographic study for the extracts at 20 and 30% of propolis and garlic for inhibition of Group 2 methicillin-resistant *Staphylococcus aureus* (MRSA) strains.

Group 2 Strains					
Extracts at 20%			Extracts at 30%		
EC50 Garlic (mg/mL)	EC50 Propolis (mg/mL)	Zt (mg/mL)	EC50 Garlic (mg/mL)	EC50 Propolis (mg/mL)	Zt (mg/mL)
33.6			33		
16.8	0.32	17.12	16.5	0.24	16.74
8.4	0.16	8.56	8.25	0.12	8.37
4.2	0.08	4.28	4.12	0.06	4.18
2.1	0.04	2.14		0.33	
	0.65				

EC50: Effective concentration 50; Zt: Sum of fractions of both extracts.

Table 3. Results of the isobolographic study (Group 1 extracts at 20 and 30%).

MRSA strain	Extracts 20%		Extracts 30%	
	EC50t	EC50e	EC50t	EC50e
X1	9.46	6.70	8.66	6.95
X8	9.46	10.90	8.66	4.70
X18	9.46	4.03	8.66	6.80
X23	9.46	4.75	8.66	4.60
X24	9.46	4.30	8.66	6.70
Average	9.46	6.13	8.66	5.95
Standard deviation (SD)	0	2.86	0	1.19

MRSA = Methicillin-resistant *Staphylococcus aureus*. EC50e = Effective experimental concentration$_{50}$; CE50t = Effective theoretical concentration (n = 5; p <0.05).

Table 4. Proportion of extracts at 20 and 30% to outline the isobologram.

Extract	Proportion of extracts at 20%	Proportion of extracts at 30%
Garlic	5.967 mg/mL	5.938 mg/mL
Propolis	0.169 mg/mL	0.012 mg/mL

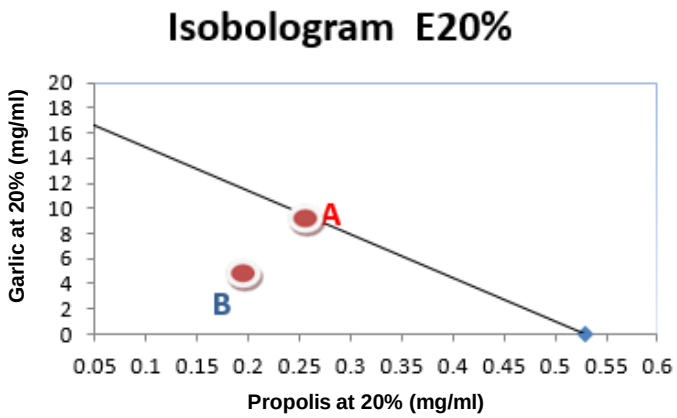

Figure 1. Isobologram of Group 1 extracts at 20%.

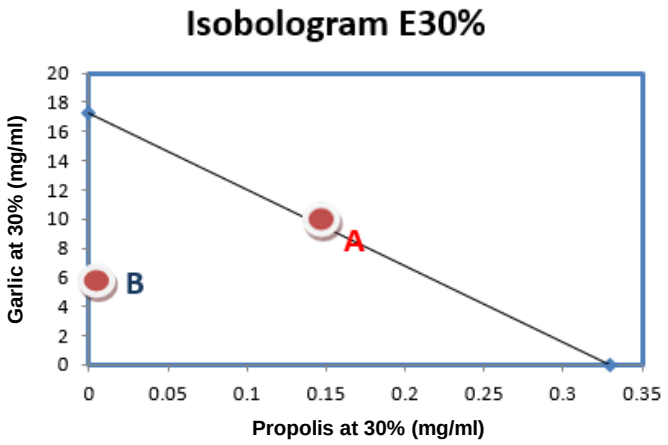

Figure 2. Isobologram of Grupo 1 extracts at 30%.

Point A represents the theoretic concentration of the combination situated on the additive line. Point B represents the experimental concentration (EC), which situates both antibiotics below the additive line. This suggests that the combination presents greater inhibitory potency than the application isolated from each antibiotic separately, which was confirmed by the determination of the interactions index, the latter less than the unit.

The isobologram was outlined based on the proportionalities of Table 4 for extracts at 30% (Figure 2), in which point A represents the theoretical (t) concentration situated on the additive line and point B, the experimental concentration (EC) found, which was situated under the additive line. This suggests that the extracts at 30% also present greater inhibitory potency than the application isolated from each antibiotic separately. To corroborate the synergistic effect observed in Figure 2, we also determined the interaction index, which also resulted less than the unit. In Group 2 strains, contrariwise, we did not find any synergistic combination, the interactions index greater than the unit in all cases.

DISCUSSION

The mechanism of activity of antimicrobal of garlic is based on the inhibition of the activity of the enzimes alkaline phosfatase, invertase, urease and papain. These enzymes are inhibited by alicine in concentrations of 0.0005 M, specially alkaline phosfatase (Lawson, 1993). On the oder hand, propolis compounds, in special flavonoids, inhibits the alkaline phosfatase too (Gutierrez, 2012). May be, both sustances, intensify the action of alicine over strains. This results in a synergistic effect.

According to the investigations carried out by Waili and colleagues in 2012 on the synergic effects of propolis extracts with ethyl alcohol on *S. aureus*, our results are in agreement that propolis utilizes the effect of another antimicrobial on being used on *S. aureus* isolates, with the difference with Waili and coworkers in 2012 which employed a second active substance: honey; on the other hand, our work highlights the effects of propolis in combination with garlic extracts, obtaining results in an interactions index of $\gamma < 1$. Huang et al. (2011) studied the synergistic combination of chitosan with silver acetate, demonstrating that they work in synergy to inhibit the *in vitro* growth of Gram-positive bacilli, methicillin-resistant *S. aureus* and Gram-negative bacteria, with MRSA strains the most resistant to such a combination. In our work, it is noteworthy that Group 2 strains demonstrated high resistance to the combination in both concentrations of the extracts, as the cited work reports.

On the other hand, Lozano and colleagues in 2014 reported a synergistic effect on applying a combination of the extracts of propolis on oregano MRSA isolates; one part of our work with garlic and propolis extracts on group 1 strains coincides with the work of the Lozano group, because it reports a synergistic effect with an interactions index of $\gamma < 1$; however, Group 2 strains showed greater resistance to the garlic and propolis extracts than to the oregano and propolis extracts.

Due to the absence of reports on combinations of garlic with other products of plant origin, it is difficult to conduct a discussion and in-depth comparison with respect to our results. However, we wish to make it clear that the bases are in order for continued investigation on alternative medicine to combat this type of microorganism. The fact that the same combinations of garlic and propolis were synergistic in the face of a group of strains while they did not present the same effect on confronting another group of these can be explained by the fact that even while they belong to the same species, there are genetic diversities between them. This was reported by Kechrid and Perez-Vazquez (2011) in a study with 236 isolates of *S. aureus*, among which 22% highly marked genetic diversity, thus a different behavior of resistance.

We can search for a possible explanation for this in the work of Krest and Keusgen (1999), in which the authors determined the enzymatic activity of allinase, which is responsible for the production of allicin in the extracts, rapidly decreasing to 42°C, indicating that the optimal

temperature of the enzyme is found between 35 and 37°C, and that inactivation occurs between 42 and 60°C; at temperatures >60°C, enzymatic activity is destroyed. It could be that at temperatures under the optimal range, their activity is also diminished; thus, our extracts were observed to be affected, given that they were worked at room temperature (20-25°C), resulting in some strains exhibiting greater resistance to garlic extracts than others.

Conclusions

Isobolographic analysis of our work suggests a synergistic effect on combining garlic and propolis in extracts at 20 and 30% on Group 1 strains. Contrariwise, possibly due to the genetic diversity of the strains studied or to other factors such as the effect of the temperature on the allinase enzyme, group 2 strains showed resistance to the combinations of the extracts.

Conflict of Interests

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

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Diversity of hydrolytic enzymes in haloarchaea isolated from Algerian sabkhas

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Algeria has numerous natural hypersaline environments (sabkha and chott) located in the north and south of the country. In the course of screening microorganisms form these environments, we isolated and characterized 44 haloarchaeal strains. According to their phenotypic characteristics and comparative 16S rRNA sequence analysis, all the isolates belonged to the family *Halobacteriaceae* including members related to species of the genera *Halorubrum*, *Haloterrigena*, *Halogeometricum*, *Halobacterium*, *Haloferax*, *Halomicrobium* and *Haloarcula*. Our finding reveals that *Halorubrum* is the most prevalent genus retrieved. The production of hydrolase was qualitatively studied on these isolates. Several strains were able to produce amylase, gelatinase and lipase. None was able to produce DNase activity. Combined hydrolytic activity was also detected in many strains.

Key words: Haloarchaea, sabkha, screening, hydrolytic enzyme.

INTRODUCTION

Halophilic microorganisms have been isolated from a range of environments: the Dead Sea, the Great Salt Lake (Utah, USA), alkaline brines of Wadi Natrun (Egypt), lake Magadi (Kenya), the hypersaline lakes of Inner Mongolia, and saline soils (Arahal et al., 1996; Grant et al., 2011; Ma and Gong, 2013). In addition, halophiles have also been isolated from highly salty foods, and the human intestinal mucosa (Abriouel et al., 2011; Lee, 2013).

The studies of these environments have shown that the diversity of microbial populations is low (Benlloch et al., 2002; Burns et al., 2004) and that, in general, microbial diversity decreases with increased salinity (Benlloch et al., 1995). The low total diversity of hypersaline environments, makes them to be taken as an ideal model system for ecological studies (Burns and Dyall-Smith, 2006). Halophilic archaea constitute the family *Halobacteriaceae* within the order *Halobacteriales*. They represent a considerable fraction of the prokaryotic world in hypersaline environments in term of number, biomass and genetic heterogeneity (Antón et al., 1999; Maturrano et al., 2006). Their adaptations to grow at high salinity concentrations make them interesting for fundamental

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research and the exploration for biotechnological process (Oren, 2010). Halophilic enzymes are unusually more stable than their normal counterparts. It has been proved that many haloenzymes are polyextremophilic. These properties made them attractive for various biotechnological applications (Kanekar et al., 2012).

Algeria has many salt lakes, located generally in semi-arid and arid regions from which salt is extracted for human consumption. Our knowledge of the diversity of halophilic microorganisms in these environments is, however, still limited; even if some investigations were performed to survey the microbial diversity of the hypersaline lakes in the Algerian Sahara (Hacène et al., 2004; Boutaiba et al., 2011). Thus, in this study, we present the taxonomic affiliation of haloarchaeal isolates recovered from three hypersaline lakes of Algeria. In addition, the production of extracellular amylase, gelatinase and lipase was investigated.

MATERIALS AND METHODS

Sampling sites

Samples used for this study were taken from three Algerian sabkhas (Ezzemoul, Melghir and Bethioua) in 2004 and 2008. These sabkhas belong to the naturally-occurring salt lakes in Algeria receiving their water supply from high ground water levels, precipitation or run-off from adjacent areas. Variations in water levels and brine concentrations occurred cyclically, starting to change in spring and the lakes completely evaporated during June-July period. Total salt concentrations and pH values were measured with refractometer (Leica) and pH-meter (Hanna), respectively.

Strain isolation and culture conditions

To perform the screening of extreme halophiles producing hydrolytic activities, samples from different sabkhas were inoculated onto two saline media. The HM contained (per litre of distilled water): 5 g proteose-peptone (Difco), 5 g yeast extract (Difco), 1 g glucose with a final total salt concentration of ca. 25% (w/v). The stock of total salts at 30% (w/v) was prepared as described by Subov (1931): 234 g NaCl, 42 g $MgCl_2.6H_2O$, 60 g $MgSO_4.7H_2O$, 1 g $CaCl_2.2H_2O$, 6 g KCl, 0.2 g $NaHCO_3$, 0.7 g NaBr and 0.005 g $FeCl_3$ and 1000 mL distilled water. Halophilic medium modified from the formulation of Oren (1983) contained (per litre distilled water): 125 g NaCl, 100 g $MgCl_2.6H_2O$, 5 g K_2SO_4, 0.1 g $CaCl_2.2H_2O$, 1 g yeast extract, 1 g casamino-acids and 2 g soluble starch. For solidification, 20 g agar L^{-1} was added.

Each medium was adjusted to pH 7.0-7.2. Portions of 0.1 mL of water samples were directly plated on solid media. Incubation was carried out in sealed plastic containers at 37°C. Colonies arising on the plates were selected for isolation based on gross morphological differential characteristics (size, pigmentation and shape). They were transferred to fresh media and pure cultures were obtained.

Phenotypic characterization

Gram staining was performed using acetic-acid-fixed samples (Dussault, 1955). NaCl tolerance was determined in growth medium supplemented with 0, 3, 5, 7.5, 10, 15, 20, 25 or 30% (w/v) NaCl. The pH dependence of growth was tested in the pH range 5.0-10.0. The temperature range for growth was determined by using incubation temperature ranging from 10 to 55°C. Catalase production was detected with 10 % (w/v) H_2O_2. The oxidase reaction was performed on filter paper moistened with a 1% (w/v) aqueous solution of N, N, N', N'-tertraméthyl-p-phenylenediamine. Tests for formation of indole and hydrolysis of starch and aesculin were performed following Gonzalez et al. (1978). The urea hydrolysis was tested according to the procedure of Larpent and Larpent-Gourgaud (1985). Reduction of nitrate was tested using the sulfanilic acid and α-naphtylamine reagent (Smibert and Krieg, 1981).

Screening for extracellular hydrolytic activities

All the isolates were tested qualitatively for the production of extracellular enzymes on plates containing 20% (w/v) total salts. The production of amylase was tested by flooding cultures on solid medium containing 1% (w/v) starch with lugol's iodine (Barrow and Feltham, 1993). Hydrolysis of gelatine and Tween 80 were tested as outlined by Gutiérrez and Gonzalez (1972). The presence of DNase activity was determined on DNase test agar. After incubation, the plates were flooded with 1 N HCl solution. Clear halos around the colonies indicated the DNase activity (Jeffries et al., 1957).

DNA extraction, 16S rRNA gene amplification and sequencing

Genomic DNA was extracted and purified from cells in the mid-logarithmic growth phase by using the method of Lind and Ursing (1986).

The 16S rRNA gene sequences were amplified by specific forward primer D30 (5'– ATTCCGGTTGATCCTGC- 3') and reverse primer D56 (5'– GYTACCTTGTTACGACTT- 3') (Arahal et al., 1996). PCR amplification was carried out as follows: denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and elongation at 72°C for 1.5 min with additional 5 s added for each cycle for a total of 30 cycles, followed by a final elongation step at 72°C for 15 min. The resulting PCR products of the expected size were purified using Microcon-100 concentrator (Amicon) and sequenced using primers given in Table 1.

Molecular identification and phylogenetic analysis

The sequences obtained were identified by a similarity based search using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012). Phylogenetic analysis was carried out using Molecular Evolutionary Genetics Analysis (MEGA) version 4.1 (Tamura et al. 2007). Multiple alignment analyses were performed with Clustal W 1.8 software (Thompson et al., 1994) selecting related sequences from the NCBI Taxonomy Homepage (Tax-Browser) database. All alignments gaps were treated as missing data. The phylogenetic trees were determined by neighbour-joining method (Saitou and Nei, 1987) and a phylogenetic consensus tree was reconstructed at random by selecting 1,000 replicates. The sequence of the type strain of *Methanospirillum hungatei* DSM 864^T was used as outgroup.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences reported in this paper have been submitted to Genbank/NCBI databases under accession numbers DQ118426; DQ120725; DQ149846; EF488827; EU409597; GQ181207-GQ181213; GQ225084-GQ225095; GQ250584; GU166402; FJ794071; FJ794073; FJ897725; GU361123-GU361125; GU361130 and GU361133-GU381143.

Table 1. Oligonucleotide primers used for PCR amplification and sequencing of bacterial and archaeal 16S rRNA genes.

Primer designation	Sequence (5'-3')	Type	Orientation
D30	ATTCCGGTTGATCCTGC	PCR, sequencing	Forward
D99	GTGTTACCGCGGCTGCTG	Sequencing	Reverse
B36	GGACTACCAGGGTATCTA	Sequencing	Reverse
D34	GGTCTCGCTCGTTGCCTG	Sequencing	Reverse
X10	ACGGGCGGTGTGTRC	PCR	Reverse
D56	GYTACCTTGTTACGACTT	PCR, sequencing	Reverse

Table 2. Geographical location and physicochemical properties of water samples.

Parameter	Hypesalines lakes		
	Ezzemoul	Melghir	Bethioua
Localisation	35°53.137´N, 6°30.200´E	34°00´ 6°07,30´	35°41´33"N, 0°18´0"O
pH	7.9	7.1	6.9
Salinity (%)	30	36	32

RESULTS

Sampling sites

Brines samples were collected from a wide geographical area. To our knowledge, this is the first microbiological study on extremely halophilic archaea from Ezzemoul, Bethioua and Melghir sabkhas. Generally, the pH is quite close to neutrality. The salinity ranged from 30 to 36% (w/v). The most saline habitat was located south (sabkha Melghir) (Table 2).

Phenotypic characterization of isolates

The assessment of the archaeal diversity of three sabkhas has been carried out through the application of cultivation methods. The isolates formed colonies ranged in color from pale-pink to red and were 1-2 mm in diameter after one week of incubation. These colonies were smooth, circular and entire. The cells were extremely pleomorphic, rod or pleomorphic-rod and stained Gram-negative. Physiological tests were performed for the isolates. All of them were extreme halophilic, had a salt concentration for growth at least 10% (w/v) NaCl, and could tolerate salt concentration up to 25-30% (w/v) NaCl. The isolates grew best between 37 and 40°C, pH of 6.5 to 7.5 and were catalase, and oxidase positive. Among the strains tested for indole production and urea hydrolysis, the majority were negative and nitrate reduction was observed within 23 isolates (Table 3).

Hydrolytic activity of isolates

The extremely halophilic strains isolated from the sabkhas were tested for their capacity to hydrolyse using extracellular enzymes substrates such as starch, gelatine, DNA and Tween 80.

Extracellular lipase activities were detected in four isolates. In contrast to the few extracellular gelatinase producers, twenty-two isolates showed extracellular amylase activities, fourteen strains from Ezzemoul sabkha. None of the isolates exhibited DNase activities. Combined hydrolytic activities were also detected in many strains. The results are summarized in Table 3 indicating that starch hydrolytic activity was predominant.

Molecular identification and phylogenetic analysis

Genomic DNA was extracted from isolates and amplified using the archaeal 16S rRNA primers. The phylogenetic analysis was done with obtained sequences and related ones obtained from the Genbank database. The 16S rRNA gene sequence similarity percentages and phylogenetic relationship are shown in Table 4 and most isolates shared more than 97% identity with their closest phylogenetic relative.

The tree constructed by neighbor-joining method depicting the phylogenetic relationships of isolates and their closest relatives is shown in Figure 1. They are placed within the family *Halobacteriaceae* (Gibbons, 1974) belonging to *Halobacteriales* order (Grant et al., 2001). The genus *Halorubrum* accounted for the majority of the isolates (59%). Among strains assigned to genus *Halorubrum*, a cluster of 20 isolates was closely related to the type strain of *Halorubrum chaoviator* Halo-G^T (Mancinelli et al., 2011). The remaining isolates were phylogenetically related to the following genera: *Halobacterium* (4 isolates), *Halogeometricum* (1 isolate),

Table 3. Phenotypic features of halophilic archaeal isolates from Algeria.

Characteristic	Strains								
	5.1	**S1**	**4**	**S7**	**K-1**	**Ez59**	**L52**	**Ez26**	**Ez228**
Pigmentation	Red	Orange	Salmon pink	Red	Orange	Pink	Pink	Orange	Red
Cell shape	Pleomorphic	Pleomorphic	Pleomorphic	Rods	Short-rods	Rods	Rods	Rods	Rods
Oxidase	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
NaCl range (%)	15-25	10-30	10-30	15-30	20-30	15-30	15-25	15-25	15-25
pH range	6.5-9.0	5.5-9.0	6.0-9.0	6.5-9.0	6.5-10	6.0-9.0	6.5-9.0	5.0-9.9	6.0-9.0
Temperature range (°C)	22-50	30-55	30-50	30-50	30-50	22-50	22-55	30-50	22-50
Urease	-	+	-	-	-	-	-	-	-
Indol production	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	-	+	+	-	-	+
Hydrolysis of:									
Aesculin	-	+	+	-	-	-	-	+	-
DNA	-	-	-	-	-	-	-	-	-
Gelatin	-	+	-	-	-	-	-	-	-
Starch	-	+	+	-	+	+	-	-	-
Tween 80	-	-	-	-	+	-	-	-	-

+, Positive; -, negative.

Table 3. Contd.

Characteristic	Strains								
	L56	**Eza4**	**Ez21**	**Ez1.2**	**Ez5-1**	**Ez5-2**	**Ez5RB**	**EzB1**	**EzB3**
Pigmentation	Pale-pink	Red	Red	Orange-red	Red	Red	Red	Red	Pink
Cell shape	Rods	Short rods	Rods	Pleomorphic rods	Rods	Rods	Pleomorphic rods	Short rods	Short rods
Oxidase	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
NaCl range (%)	15-25	15-25	15-30	15-25	15-30	15-25	10-30	10-30	10-30
pH range	6.5-9.0	6.0-9.0	5.5-8.5	7.0-9.0	6.5-9.0	6.5-9.0	6.5-9.0	7.0-9.0	6.5-9.0
Temperature range (°C)	30-55	22-50	30-55	30-55	22-50	22-50	30-50	30-50	30-50
Urease	-	-	+	-	-	-	-	-	-
Indol production	-	+	-	-	-	-	-	-	-
Nitrate reduction	+	-	+	+	-	-	-	-	+
Hydrolysis of:									
Aesculin	-	+	-	+	+	+	-	-	-
DNA	-	-	-	-	-	-	-	-	-
Gelatin	+	-	+	+	-	-	-	-	-
Starch	-	+	-	+	+	+	+	+	+
Tween 80	-	-	-	-	-	-	-	-	-

Haloterrigena (3 isolates), *Haloferax* (4 isolates), *Halomicrobium* (4 isolates) and *Haloarcula* (4 isolates).

DISCUSSION

Sabkhas and chotts are examples of high salty environments inhabited by halophilic microorganism. Our study is the first attempt to investigate halophilic archaea in the sabkhas of Ezzemoul, Bethioua and chott Melghir.

Throughout the course of this work, we further characterized 44 isolates. All were contained within the family *Halobacteriaceae*, a typical and dominant group in

Table 3. Contd.

Characteristic	Strains								
	EzS2	**EzS6**	**Ez522**	**Ez526**	**Ez24**	**EzA1**	**Sm**	**A**	**Beja5**
Pigmentation	Red	Red	Red	Pink	Orange	Red	Pale-pink	Red	Pale-pink
Cell shape	Rods	Rods	Short rods	Rods	Pleomorphic	Rods	plleomorphic	Rods	Pleomorphic
Oxidase	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
NaCl range (%)	15-30	15-30	12.5-30	15-30	10-30	15-30	10-30	20-30	12.5-30
pH range	6.5-9.0	6.5-9.0	6.0-8.5	6.5-9.0	6.5-8.5	6.0-8.5	6.5-8.5	6.5-9.0	6.0-9.0
Temperature range (°C)	30-50	30-50	22-30	22-50	30-50	22-50	22-50	30-50	22-50
Urease	-	-	-	-	-	-	-	-	-
Indol production	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	+	-	-	-	+	+	-
Hydrolysis of:									
Aesculin	-	-	+	+	-	-	-	-	+
DNA	-	-	-	-	-	-	-	-	-
Gelatin	-	-	-	-	-	-	-	-	-
Starch	+	-	+	-	-	+	-	-	-
Tween 80	-	-	-	+	+	-	-	-	-

Table 3. Contd.

Characteristic	Strains								
	bejS3	**bej51**	**KL**	**MGG2**	**MGG3**	**MG23**	**MG25**	**MG215**	**MG525**
Pigmentation	Beige-orange	Pink	Orange	Red-orange	Orange	Pink	Pink-red	Orange	Red
Cell shape	Rods	Pleomorphic	Pleomorphic	Pleomorphic	Rods	Short rods	Rods	Pleomorphic rods	Short rods
Oxidase	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
NaCl range (%)	10-25	10-25	10-25	10-30	10-30	10-30	10-25	15-30	10-30
pH range	6.0-8.5	6.5-9.0	6.0-9.0	5.5-9.0	6.0-9.0	6.5-9.0	5.5-8.5	6.5-9.0	5.5-8.5
Temperature range (°C)	22-50	30-50	30-55	22-50	22-50	30-50	22-50	30-50	30-55
Urease	-	-	-	+	+	-	-	-	-
Indol production	+	-	-	-	-	+	+	+	-
Nitrate reduction	+	+	+	+	+	-	-	-	+
Hydrolysis of:									
Aesculin	**-**	**-**	**-**	**+**	**+**	**-**	**-**	**-**	**+**
DNA	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Gelatin	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
Starch	**+**	**+**	**-**	**+**	**+**	**-**	**-**	**-**	**-**
Tween 80	**-**	**+**	**-**	**-**	**-**	**-**	**-**	**-**	**-**

hypersaline environments by cultivation methods. We discovered seven genera: *Halorubrum*, *Halobacterium*, *Haloferax*, *Halomicrobium*, *Haloarcula*, *Haloterrigena*, *Halogeomtricum* and two novel species (Kharroub et al., 2006, 2008). The haloarchaea were characterized by their obligate halophilic lifestyle and their aerobic heterotrophic metabolism. The sequence similarities of isolates ranged between 95.8 and 99.8% to closely related species. The majority of the strains from this study were more closely related to *H. chaoviator* Halo-G^T isolated from sea salt in Baja California, Mexico, Western Australia and the Greek island of Naxos (Mancinelli et al.,

Table 3. Contd.

Characteristic	Strains							
	MG526	Set21	KM	Bet 58	Bet25	Bet213	Bet217	Bet512
Pigmentation	Pink-red	Red	Red-orange	Red-orange	Pale-pink	Pink	Orange	Orange
Cell shape	Short rods	Short rods	Rods	Rods	Pleomorphic	Short rods	Rods	Rods
Oxidase	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
NaCl range (%)	15-30	10-25	15-30	15-30	15-30	10-30	10-30	10-30
pH range	6.0-9.0	6.0-8.5	6.0-9.0	6.0-9.0	6.0-8.5	6.0-9.0	6.5-8.5	6.0-8.5
Temperature range (°C)	22-50	30-55	30-55	22-50	30-55	30-55	30-50	22-55
Urease	-	-	-	-	-	-	-	+
Indol production	+	+	-	-	+	-	-	-
Nitrate reduction	-	+	+	+	-	-	-	+
Hydrolysis of:								
Aesculin	+	-	+	+	+	+	+	-
DNA	-	-	-	-	-	-	-	-
Gelatin	-	+	-	-	+	-	+	-
Starch	-	+	-	+	-	+	-	+
Tween 80	-	-	-	-	-	-	-	-

Table 4. Affiliations of the haloarchaeal 16S rRNA gene sequences of the basis pairwise comparison by the Ez Taxon server 2.1.

Strain	Isolation site	Accession no.	Identity (%)	Taxon (type strain)
5.1[a]	S1	DQ118426	100	*Halorubrum ezzemoulense* (DQ118426)
S1	S1	DQ120725	96.5	*Haloarcula salaria* HST01-2R^T (FJ429317)
4	S1	DQ149846	95.8	*Haloferax prahovense* TL6^T (AB258305)
S7	S1	EF488827	99.4	*Halorubrum chaoviator* Halo-G^T (AM048786)
K-1[a]	S1	EU409597	100	*Halomicrobium katesii* Al-5^T ((EF533994)
Ez59	S1	GQ181207	99	*Halorubrum chaoviator* Halo-G^T (AM048786)
L52	S1	GQ181208	99.7	*Haloterrigena thermotolerans* PR-5^T (AF115478)
Ez26	S1	GQ181209	99.5	*Halorubrum chaoviator* Halo-G^T (AM048786)
Ez228	S1	GQ181210	99.5	*Halorubrum chaoviator* Halo-G^T (AM048786)
L56	S1	GQ181211	99	*Halorubrum chaoviator* Halo-G^T (AM048786)
Eza4	S1	GQ181212	99.2	*Halorubrum californiense* SF3 213^T(EF139654)
Ez21	S1	GQ181213	98.2	*Halobacteriumjilantaiense* NG4^T (DQ256409)
Ez1.2	S1	GQ225084	98.9	*Haloarcula quadrata* 801030^T (AB010964)
Ez5-1	S1	GQ225086	98.9	*Halorubrum chaoviator* Halo-G^T (AM048786)
Ez5-2	S1	GQ225087	99.1	*Halorubrum chaoviator* Halo-G^T (AM048786)
Ez5RB	S1	GQ225088	99.7	*Halorubrum chaoviator* Halo-G^T (AM048786)
EzB1	S1	GQ225089	99.8	*Halorubrum chaoviator* Halo-G^T (AM048786)
EzB3	S1	GQ225090	97.5	*Haloterrigena thermotolerans* PR-5^T (AF115478)
EzS2	S1	GQ225091	98.8	*Halorubrum chaoviator* Halo-G^T (AM048786)
EzS6	S1	GQ225092	99.8	*Halorubrum chaoviator* Halo-G^T (AM048786)
Ez526	S1	GQ225094	99	*Halorubrum californiense* SF3 213^T(EF139654)
Ez24	S1	GQ225095	99.4	*Halorubrum chaoviator* Halo-G^T (AM048786)
EzA1	S1	GQ250584	99.1	*Halorubrum chaoviator* Halo-G^T (AM048786)
EzSm	S1	GU166402	99.1	*Haloterrigena thermotolerans* PR-5^T (AF115478)
EzA	S1	GQ225085	99.7	*Halobacterium salimarum* NRC-1 (AE004437)
beja5	S2	GU361123	98.5	*Halorubrum chaoviator* Halo-G^T (AM048786)
bejS3	S2	GU361124	98.9	*Haloferax lucentense* JCM 9276^T (AB081732)
bej51	S3	GU361125	97.8	*Haloferax prahovense* TL6^T (AB258305)

[a]Strain described as new species.

Table 4. Contd.

Strain	Isolation site	Accession no.	Identity (%)	Taxon (type strain)
KL	S2	FJ794071	98.4	*Halogeometricum rufum* R01-4^T (EU887286)
MGG2	S3	GU361137	98	*Haloarcula marismortui* ATCC43049^T (AY596298)
MGG3	S3	GU361138	98	*Haloarcula vallismortis* CGMCC1.2048 rrnB (EF645688)
MG23	S3	GU361139	99.8	*Halorubrum chaoviator* Halo-G^T (AM048786)
MG25	S3	GU361140	98.6	*Halorubrum chaoviator* Halo-G^T (AM048786)
MG215	S3	GU361141	99.6	*Halorubrum chaoviator* Halo-G^T (AM048786)
MG525	S3	GU361142	98.3	*Halorubrum chaoviator* Halo-G^T (AM048786)
MG526	S3	GU361143	99.2	*Halorubrum californiense* SF3 213^T(EF139654)
Set21	S3	GU361130	99.5	*Haloferax lucentense* JCM 9276^T (AB081732)
KM	S2	FJ794073	96.6	*Halomicrobium katesii* Al-5^T (EF533994)
Bet 58	S2	FJ897725	97.9	*Halomicrobium katesii* Al-5^T (EF533994)
Bet25	S2	GU361133	98.8	*Halorubrum chaoviator* Halo-G^T (AM048786)
Bet213	S2	GU361134	96.8	*Halomicrobium katesii* Al-5^T (EF533994)
Bet217	S2	GU361135	99.1	*Halorubrum californiense* SF3 213^T(EF139654)
Bet512	S2	GU361136	99.5	*Halorubrum chaoviator* Halo-G^T (AM048786)

S1, Ezzemoul sabkha; S2, Bethioua sabkha; S3, Chott Melghir.

2011). Such dominance of the genus *Halorubrum* is in agreement with previous cultivation-based study on halophilic archaeal communities inhabiting hypersaline environments (Burns et al., 2004; Xu et al., 2007; Trigui et al., 2011; Chen et al., 2013). The genus, *Halorubrum* contains the largest number of species (currently 27 species) and was among the most frequently revealed and probably ubiquitous archaeon in different hypersaline lakes that were confirmed by several diversity studies (Pašić et al., 2007; Manikandan et al., 2009). The predominant population tends to be made up of strains belonging to the genera *Natrinema* and *Haloferax* found in Rambla Salada (Spain) (Luque et al., 2012); *Halorubrum* and *Haloferax* in solar salterns of Tamil Nadu (India) (Manikandan et al., 2009); *Halobacterium* in Ocnei hypersaline lake (Romania) (Baricz et al., 2014). Compared to several lakes, at genus level, almost the same rate (6 and 7 genera) is observed (Pašić et al., 2007; Tsiamis et al., 2008).

The culture media applied in this study had varying degrees of selectivity on the detected species. Some of the isolates were able to reduce nitrate to nitrite, which suggests that they might be involved in the nitrogen cycle within these environments. A survey of the literature on the taxa of haloarchaea showed that some strains have been reported to possess the urease activity (Mizuki et al., 2005). In the present work, only four strains affiliated to genus *Haloarcula* were shown to be urease producers.

Most research studies performed on hypersaline environments have focused on the microbial diversity and on ecology. However, the studies based on the diversity of halophilic microorganisms showing hydrolytic activities in hypersaline habitats remain unexplored. It is interesting to emphasize that multiple hydrolytic activity was detected in the isolates of this study supporting previous studies in other hypersaline environments. Many species of the family *Halobacteriaceae* produce hydrolases such as proteases, lipases, amylase and amyloglucosidases that function at high salinity (Pérez-Pomares et al., 2003; Oczan et al., 2009; Siroosi et al., 2014). The most recurrent hydrolytic activity detected in our study was amylase (50% of total isolates), in agreement with those found by several authors (Moreno et al., 2009; Makhdoumi Kakhki et al., 2011). Several halophilic enzymes also function at high temperatures (Vidyasagar et al., 2006; Moshfegh et al., 2013). Some archaeal enzymes are of potential interest, such as amylase of *Haloarcula* sp. (Fukushima et al., 2005) and β-galactosidase of *Halorubrum lacusprofundi* (Karan et al., 2013). Thus, the hypersaline environments represent a valuable source of extracellular hydrolytic enzymes with potential in different economical fields (DasSarma et al., 2010; Delgado-Garcia et al., 2012; Schreck and Grunden, 2014).

A general conclusion that emerged from this study is that the diversity of halophilic archaea described above is undoubtedly a small fraction of the true diversity of halophilic archaea in Algerian hypersaline environments. These environments could be used as a starting point, for more cultivation attempts. Also, their localization in semi-arid and arid regions where solar heating, especially in the chott Melghir, results in temperature up to 50°C in summer. These conditions make these habitats a good potential for halothermophiles prokaryotes.

Conflict of Interests

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

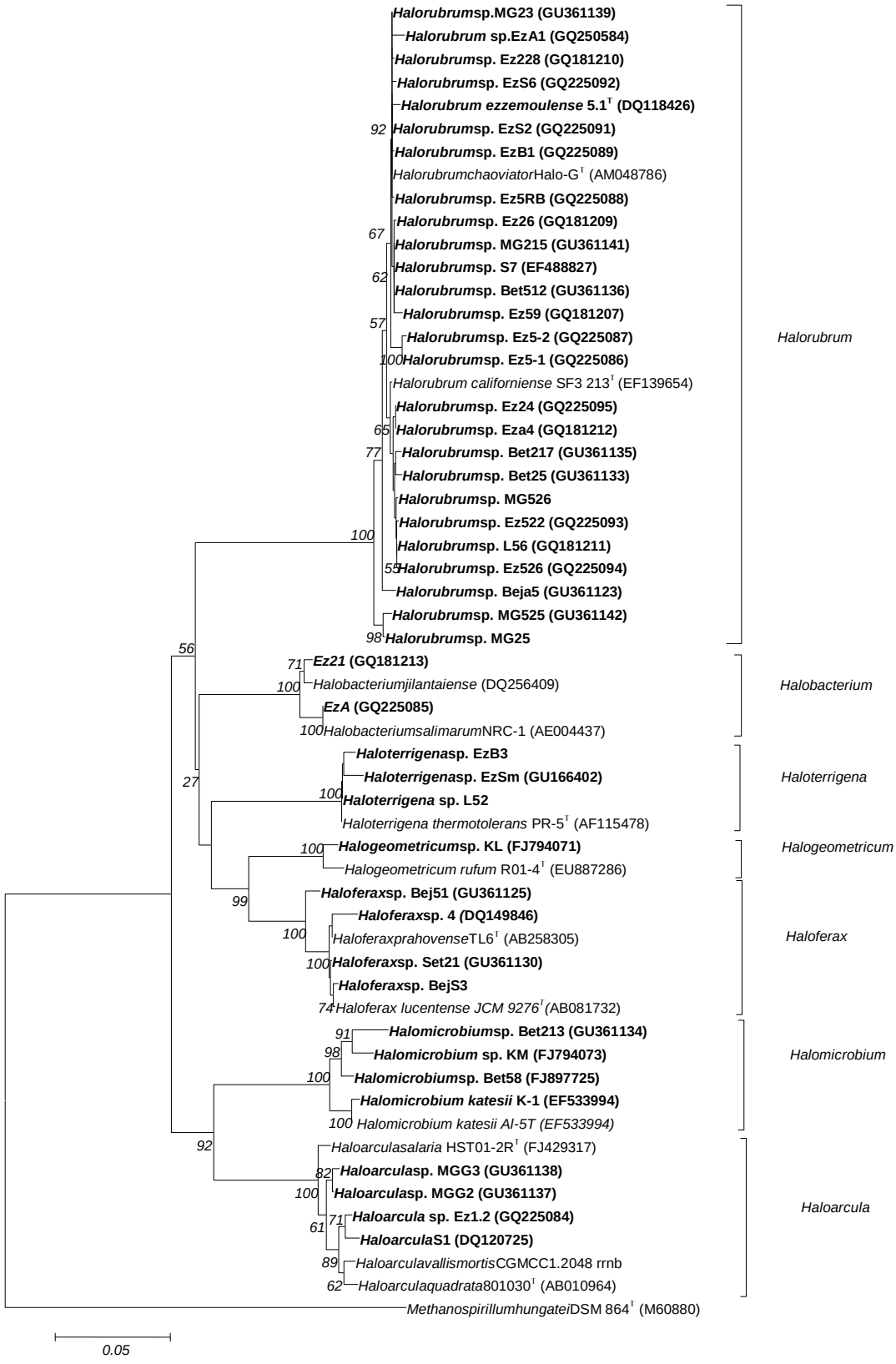

Figure 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strains with respect to other species of the family *Halobacteriaceae*. The 16S rRNA gene sequence of *Methanospirillum hungatei* DSM 864^T was used as outgroup. Numbers at branch points indicate the level of bootstraps support, based on 1,000 resamplings.

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Full Length Research Paper

Screening of *Piper hispidum* endophytic fungi that produce terpenes and antibacterial substances

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The plant species *Piper hispidum* has extensive economic potential due to the production of safrole, a component of its essential oil with proven antimicrobial and insecticidal activity. One strategy for obtaining bioactive compounds through extraction from plant species is by using endophytic microorganisms, since they can produce the same substances synthesized by the host. Therefore, this study aimed to isolate fungal endophytes from *P. hispidum* and verify their ability to produce terpenes and antimicrobial substances. Fifty-eight (58) endophytic fungi were investigated. In the metabolic media compounds with antimicrobial activity against *Staphylococcus aureus*, *Proteus vulgaris*, *Escherichia coli* and *Shigella sonnei* were detected; none of the fungi produced safrole. However, the results suggest the production of β-caryophyllene and terpinolene by three isolates. This study shows that investigation of fungal diversity associated with *P. hispidum* offers promising perspectives for biotechnology.

Key words: *Piperaceae*, Amazon fungi, high performance liquid chromatography (HPLC), essential oils, antibacterial activity.

INTRODUCTION

In plants, there are three major groups of secondary metabolites: phenolic compounds, alkaloids and terpenes. Terpenes are formed from mevalonic acid or from the reaction with pyruvate and glyceraldehyde 3-phosphate (Goodwin, 1964). In fungi, the induction for accumulation (or biosynthesis) of terpenes may occur through an alternative route to mevalonic acid, the methylerythritol phosphate pathway. This compound is derived from pyruvate and glyceradehyde 3-phosphate, originating from the degradation of glucose (Zhi-Iin et al., 2007).

The components of essential oils, complex natural mixtures which can contain about 20 to 60 components at quite different concentrations, include two groups of distinct biosynthetical origin. The main group is composed of terpenes and terpenoids and the other of

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aromatic and aliphatic constituents, all characterized by low molecular weight. These compounds have today an important role in several industries, since their use as raw materials has become indispensable for many products with high added value (Bakkali et al., 2008). These oils are used in cosmetics, perfumery and pharmacy, in the food industry, in disinfectants, soaps, plastics, paints, rubber and insecticides, among others (Robles and Garzino, 1998; Rozenbaum et al., 2006; Bakkali et al., 2008; Maia and Andrade, 2009). In particular, the antimicrobial activity has been considered the basis for various applications of essential oils including for food preservation and alternative pharmaceutical manufacturing (Bakkali et al., 2008).

The terpenes within the essential oils cover a wide variety of substances of vegetable origin and their ecological importance in the plant defense system is well established. They have also been widely reported to possess antimicrobial activity. The essential oil of *Satureja montana* L., rich in the monoterpene geraniol, has inhibited the growth of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli, Pseudomonas aeruginosa* and *Bacillus subtilis* (Cávar et al., 2008). Furthermore, many essential oils and compounds of these isolates have been recently recognized as powerful natural antioxidants, which could be used as replacements for synthetic antioxidants (Bozin et al., 2006). The monoterpene terpinolene, present in small amounts in the essential oils of *Pinus mugo*, *Manilla elemi*, *Nectandra elaiophora* and *Dacrydium colensoi*, among other species, effectively prevents the oxidation of low density lipoprotein (LDL) (Graßmann et al., 2005), shows antiviral activity, and acts against pathogenic fungi (Opdyke, 1976). The β-sesquiterpene caryophyllene has been associated with chemical communication between species. This volatile terpenoid is produced in maize roots when attacked by insects, attracting nematodes that parasitize the larvae of insects (Gershenzon and Dudareva, 2007). Moreover, β-caryophyllene has different interesting biological activities, including anticancer activity (Calixto, 2000), and has been found to be produced by microorganisms (Strobel et al., 2007; Strobel et al., 2011).

Piper hispidum L., or pepper jack, occurs naturally in the Amazon and has attracted great interest due to the production of essential oil rich in safrole, which has effective action in the control of pathogens such as fungi and bacteria, and proven anti-inflammatory and analgesic action with low toxicity (Maia and Andrade, 2009).

Over the last years, evidences have justified the interest on endophytic fungi, defined as those that live asymptomatically in the apoplastic spaces or within the plant cells, at least during a significant part of their life cycle (Petrini, 1991). These microorganisms can be ecologically important to their host, sometimes giving them support, and other times being the protagonists in fundamental processes of plant survival (Artursson et al., 2006; Yue et al., 2000). Some endophytes can produce similar or identical biologically active constituents as the host (Kusari et al., 2014), such as taxol (Stierle et al. 1993). In addition, many fungal endophytes produce secondary metabolites and some of these compounds exhibit antibacterial activity that strongly inhibits the growth of other microorganisms (Gunatilaka, 2006, Tayung et al., 2012). Therefore, in this study endophytic fungi associated with *P. hispidum* were isolated and the metabolites produced by these microorganisms were analyzed, in order to identify fungi that may produce terpenes and compounds with antibacterial activity.

MATERIALS AND METHODS

Isolation of endophytes

The roots, stems and leaves of *P. hispidum* were collected in the urban area of Manaus (Specimen 1 was located at 3°3'4.42"S and 60°4'0.32"W; Specimen 2 was located at 3°6'17.31"S and59°59'27.57"W), during the raining season, for the isolation of the endophytes. The surface of plant fragments of *P. hispidum* was disinfected in 70% ethanol for 2 min, 2% sodium hypochlorite for 2.5 min, 70% ethanol for 1 min, and finally washed with sterile distilled water for 3 min (Banhos et al., 2014). In this specific situation, the water was plated and incubated at 26°C as a control of sterilization procedure. The tissues were then cut into fragments of approximately 0.5 cm and transferred to dishes containing PDA (potato-dextrose-agar) media (Himedia, India) supplemented with tetracycline. The plates were incubated at 28°C for 7 days. The streaking purification technique was used to obtain isolated colonies. After allowing the isolates to grow, the frequency of isolation in the different tissues was calculated, expressed as colonization rate (CR), using the relation:

$$CR = \frac{number\ of\ \ plant\ \ fragments\ with\ \ fungal\ growth}{total\ \ number\ of\ \ plant\ \ fragments}$$

The endophytic fungi isolated from *P. hispidum* were stored using the method proposed by Castellani (1939). All isolated endophytic fungi were deposited into the Collection of the Laboratory of the School of Health Sciences, State University of Amazonas (ESA/UEA).

Obtainment of secondary metabolites

Five millimeter diameter discs of the PDA media (Himedia, India) containing fungi mycelia (previously grown on PDA for 7 days at 28°C) were transferred into 150 mL Erlenmeyer flasks with 50 mL of PD (potato-dextrose) liquid media supplemented with 0.2% yeast extract under sterile conditions, according to the method described by Banhos et al. (2014) with modifications. The flasks were incubated without agitation for 14 days at a temperature of 28°C. The culture broth was then vacuum filtered to separate the mycelia. The filtered metabolic broth was analyzed to verify the production of terpenes and antibacterial activity.

Evaluation of the production of essential oils

The metabolic broth was filtered through a 45 µm membrane (Millipore, Brazil) and analyzed by HPLC (Varian® ProStar 310)

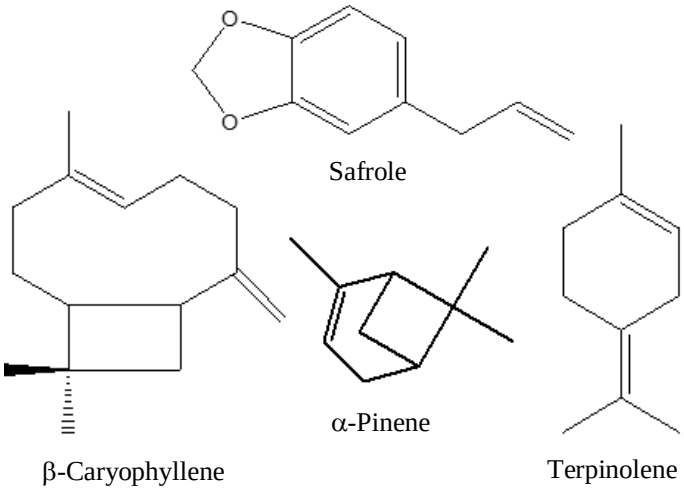

Figure 1. Chemical structure of safrole and the terpenes β-caryophyllene, α-pinene and terpinolene, present in the metabolites of endophytes from *Piper hispidum.*

Table 1. Antibiotic susceptibility presented by different bacterial strains used in this work.

Bacterial species	Antibiotic
Staphylococcus aureus	VAN (MIC < 4 mg/L)
Escherichia coli	CHL / CAR (MIC < 4 mg/L)
Shigella sonnei	STR (MIC < 4 mg/L)
Proteus vulgaris	CAR / STR (MIC < 4 mg/L)

VAN, vancomycin; CH, chloramphenicol; CAR, carbenicilin; STR, streptomycin; GEN, gentamicin; MIC, minimum inhibitory concentration.

using a C18 column (250 x 4.6 mm), UV-Vis detector at 257 nm and a acetonitrile/water gradient as the eluent (up to 5 min H_2O/ACN 50:50; from 5 to 35 min 100% ACN). The injections were performed in triplicate. The chromatograms obtained for the metabolic broths were compared with the chromatograms obtained for standard compounds: β-caryophyllene (98%, Aldrich, United States), α-pinene (98%, Fluka, Germany); safrole (97%, Sigma, United States) and terpinolene (85%, Fluka, Germany). These compounds have been reported as main constituents of the essential oil of *P. hispidum,* and its chemical structures are shown in Figure 1.

Antimicrobial activity assay

The metabolic broths were used for *in vitro* antibacterial tests against pathogenic bacteria. Strains of *Staphylococcus aureus*, *Escherichia coli* (O157: H7), *Proteus vulgaris* and *Shigella sonnei* were kindly provided by the Tropical Virology Laboratory of the National Institute for Amazonian Research - INPA. Bacterial cultures were kept in nutrient agar at 36°C. The bacterial strains were previously tested regarding its antibiotic susceptibility (NCCLS, 2007), as showed in Table 1.

A bacterial suspension was obtained in saline solution (6 $x10^8$ CFU/mL) and with the aid of a swab, the suspension was inoculated on Mueller Hinton agar (MHA, Himedia, Mumbai, India) over the whole plaque. Small wells with 0.5 cm of diameter were made in each plate in order to add 100 µL of the metabolic broth. For the negative controls, the wells were filled with potato dextrose broth and tested against the bacterial strains. All assays were performed in triplicate, and results were considered consistent only when the three replicates presented the same result.

The plates were incubated aerobically for 18-24 h at a temperature of 35-37°C. The metabolic broths which indicated positivity for the inhibition of any of the test microorganisms were analyzed by high performance liquid chromatography (HPLC) in order to compare their chromatographic profile with that of a commercial antibiotic (Amoxicillin). A Varian ® ProStar 310 chromatograph was used, equipped with a C18 column (250 x 4.6 mm) and UV-Vis detector at 257 nm, and acetonitrile/sodium phosphate buffer 25 mM pH 3.0 (20:80) was employed as the eluent. The injections were performed in triplicate.

RESULTS AND DISCUSSION

Isolation of endophytes from *P. hispidum*

From the two specimens of *P. hispidum*, 120 endophytic fungi were isolated using 25 fragments of each plant tissue (roots, stems and leaves). After separation of the isolates into nine morphological groups (Barnett and Hunter, 1972), 58 isolates were selected for the subsequent stages, which represent the macro-morphological diversity of the endophytic fungi isolated from *P. hispidum*. The growth of a greater number of endophytes from the leaves of each *P. hispidum* specimen was noted, as shown in Table 2.

Wilson (1996) suggests that there are two major possibilities for fungal dissemination within the host plant: vertical transfer, when fungi are transmitted through the seeds, and horizontal transfer, when fungal colonies are passed from plant to plant, via spores. Considering the horizontal dissemination of fungi and the milder asepsis performed on leaves when compared with roots and stems, it is possible to understand the obtainment of a greater number of fungal isolates from the leaves of *P. hispidum*. Moreover, the stem and stalk usually present a greater resistance to changes in the natural plant habitat, thus offering a less favorable environment for the flux of microorganisms in these parts of the plant.

Colonization rates (CRs) were higher than 0.5 for all plant parts (Table 2), demonstrating that the technique used for the isolation of endophytic fungi was appropriate, enabling the acquirement of a significant number of fungal isolates from the two specimens of *P. hispidum.* Fungi were present in all cultivated fragments of specimen 2 leaves (CR = 1.00). For the stems and roots, the CR values remained close to those of specimen 1.

Specimens 1 and 2 had CR values of 0.81, and 0.79, respectively, indicating that all fragments inoculated from these specimens only 19 and 21%, respectively, showed no fungal growth. This finding may be related to the diversity of interactions of the specimens of *P. hispidum* with other plants in the region where they are grown,

Table 2. Number of isolated and selected fungi, and the colonization rate (CR) of *P. hispidum* specimens.

Sample	Code[a]	Isolated fungi	Selected fungi	Colonization rate
	PH-L	22	13	0.88
Specimen 1	PH-S	18	8	0.72
	PH-R	19	6	0.76
Total		59	27	0.79
	PH-L	25	14	1.00
Specimen 2	PH-S	21	10	0.84
	PH-R	15	7	0.60
Total		61	31	0.81

[a]Identification code for the isolates consists of the initials of the plant, PH, followed by the letter that represents the tissue from which it was removed (L - leaf, S - stem, and R - root).

Table 3. Retention times obtained for the major peaks of commercial standards analyzed by reverse phase HPLC.

Standard	Commercial source	Retention time[a] (min)
β-Caryophyllene	Aldrich, 98%	14.28
α-Pinene	Fluka, 98%	9.05
Safrole	Sigma, 97%	13.11
Terpinolene	Fluka, 85%	14.38

[a]Retention times obtained on a Varian® ProStar 310 chromatograph with C18 column (250 x 4.6 mm), UV-Vis detector at 257 nm and acetonitrile/water gradient as eluent (up to 5 min H_2O/ACN 50:50; from 5 to 35 min 100% ACN).

since vegetation type, plant age, and seasonal difference can change the diversity and abundance of endophytes (Yang and Dai, 2013).

The factors affecting endophyte community structure have been explored in many researches. Arnold and Lutzoni (2007) reported that the diversity of endophytes at both the individual and plant community levels increased with decreasing latitude (from poles to equator). Furthermore, they also found that endophytes isolated within a specific biogeographic zone (arctic, temperate or tropical) were often absent from other zones. According to Baynes et al. (2012), at the local level, other factors are operative, such as water availability, temperature, agricultural chemicals, and plant metabolites. Pimentel et al. (2006) found qualitative and quantitative differences in the type and number of soybean isolates obtained from greenhouse and field-grown plants, with more isolates being obtained from the latter. Saunders and Kohn (2009) demonstrated that production of plant defense compounds influenced the endophyte community within maize, and variable leaf chemistry can explained differences in endophyte communities among host species (Arnold and Herre, 2003).

In a recent study, Yang and Dai (2013) investigated interactions between endophytic fungi infecting the same host, *Atractylodes lancea*, an essential oil producer. The authors verified that the levels and components of essential oils affect the growth of endophytes, and high concentrations of these metabolites suppress the growth of endophytes, which have the capacity to degrade and biotransform essential oils.

Production of essential oil compounds by *P. hispidum* endophytes

In general, it could be noted that the chromatographic profiles obtained for the metabolic broths present two groups of molecules of different polarities, since reverse phase HPLC was used as analytical method. The first group consists of polar molecules, which have shorter retention times, (0 to 5 min). The second group contains less polar molecules, with retention times of between 10 and 20 min.

When submitted to chromatographic analysis, the commercial standards of safrole, β-caryophyllene, α-pinene and terpinolene presented major peaks with the

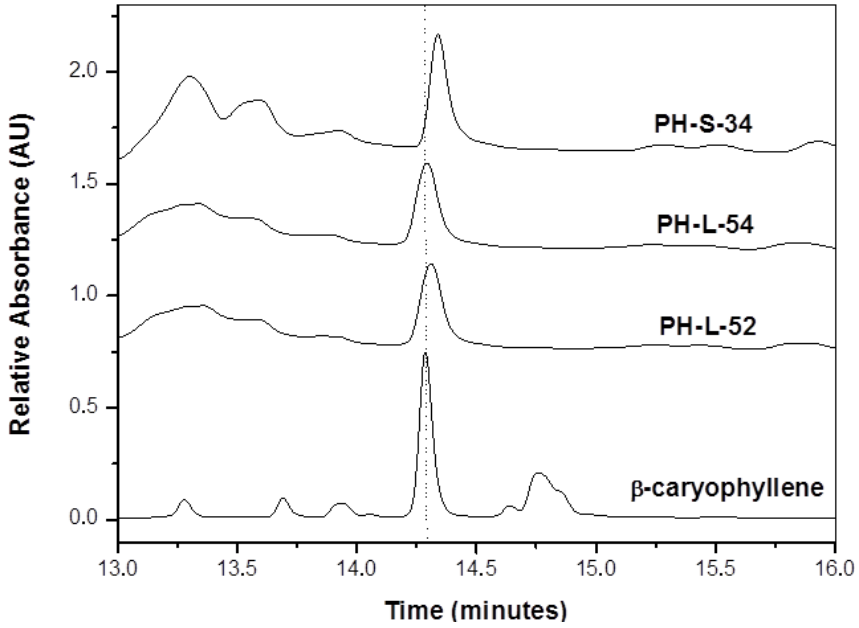

Figure 2. Chromatograms of β-caryophyllene, and the metabolic broths of endophytes PH-L 54, PH-L 52 and PH-S 34 isolated from *Piper hispidum.*

retention times shown in Table 3.

Safrole is a natural allylbenzene of wide distribution in the plant kingdom. The major component in the essential oil of *P. hispidum* (Wadt et al., 2004; Oliveira et al., 2007) had a characteristic peak at a retention time of 13.11 min. Surprisingly, the presence of safrole was not observed within the metabolic broth of the endophytic fungi selected in this study.

α-Pinene, found at a concentration of 10.2% in *P. hispidum* (Bottia et al. 2007), showed a major peak at 9.05 min. In the chromatogram obtained for the metabolites of the isolated endophytes, any peak with this retention time was not observed. Therefore, the isolates investigated in this study do not produce this terpene.

β-Caryophyllene is present at low concentrations in the essential oil of *P. hispidum* (Bottia et al., 2007). As can be seen in Figure 2, the terpene standard showed a major peak in the chromatogram at 14.28 min.

It can be noted in the chromatograms of Figure 2 that the metabolic media of the isolated endophytes PH-L 54, PH-L 52 and PH-S 34 show peaks centered at 14.29, 14.31 and 14.33 min, respectively. The retention times of all peaks are close to that observed for β-caryophyllene (14.28 min). These results suggest that there is the possibility that the isolated endophytes PH-L 54, PH-L 52 and PH-S 34 produce β-caryophyllene. However, due to the proximity of the retention times, PH-L 54 was identified as the prime candidate for the production of β-caryophyllene. The other fungal metabolic broth analyzed by HPLC showed no peaks with retention times similar to those of the spikes of β-caryophyllene.

Terpinolene has been consistently isolated from plants of the genus *Piper*. However, it is found in low concentrations (1.2%) in plants of the species *P. hispidum* (Bottia et al., 2007). The metabolic broth of the strain PH-L 52 showed a peak with a retention time similar to the major peak of terpinolene, as can be seen in the chromatograms of Figure 3. The presence of peaks centered at 14.29, 14.32 and 14.37 min can be noted in the chromatograms of the metabolic broths of PH-L 54, PH-L 52 and PH-S 34, respectively, which are very close to the major peak in the chromatogram of the terpinolene standard (14.38 min). Thus, there is the possibility that PH-L 54, PH-L 52 and PH-S 34 produce terpinolene. However, PH-S 34 was considered to be the most promising candidate for the production of terpene, since its metabolic broth shows a peak with a retention time closest to the peak of terpinolene. The other fungal extracts analyzed by HPLC showed no peaks with retention times similar to that of terpinolene. Thus, only these three strains could produce this terpene as a secondary metabolite.

The production of terpenes by endophytic fungi has been widely explored. Souza et al. (2011) reviewed the production of terpenoids by endophytic fungi and their biological activities, in the period of 2006 to 2010. Sixty five sesquiterpenes, 45 diterpenes, five meroterpenes and 12 other terpenes, amounting to 127 terpenoids were isolated from endophytic fungi.

Strobel et al. (2011) reported that a *Phoma* sp. isolated and characterized as endophytic and as a pathogen of *Larrea tridentata* produces a mixture of volatile organic compounds (VOCs), including a series of sesquiterpenoids.

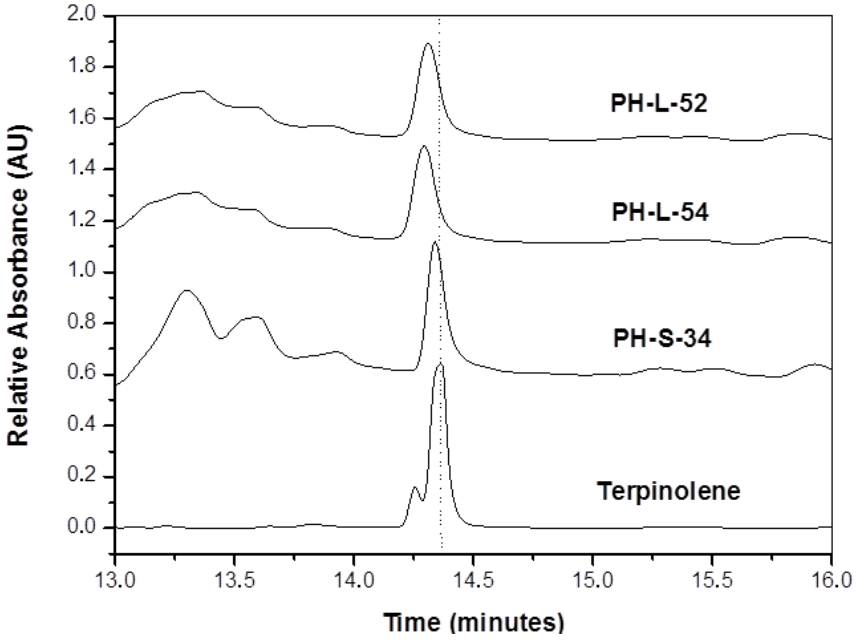

Figure 3. Chromatograms of terpinolene and the metabolic broths of endophytes PH-L 54, PH-L 52 and PH-S 34 isolated from *Piper hispidum.*

Trans-caryophyllene, a product in the fungal VOCs, was also noted in the VOCs of the host plant. Besides, the authors verified that the gases of *Phoma* sp. possess antifungal properties and is markedly similar to that of a methanolic extract of the host plant. The terpene caryophyllene was also produced by the fungus *Muscodor albus* E-6, an endophyte of *Guazuma ulmifolia* (Strobel et al., 2007).

The production of terpenes was also reported for an endophytic fungus isolated from *P. aduncum*. Silva et al. (2010) isolated two new presilphiperfolane sesquiterpenes from the ethyl acetate extract of *Xylaria* sp., obtained from the leaves of *P. aduncum*, along with two known eremophilane sesquiterpenes, phaseolinone and phomenone, which displayed cytotoxic and antifungal activities.

Production of antimicrobial compounds

The metabolic media of endophytic fungi isolated from *P. hispidum* were used in the tests for *in vitro* antagonism against pathogenic bacteria. Of the total number of metabolic media tested, 15 (25.9%) had antagonistic activity against one or more test bacteria, as can be observed in the data in Table 4.

The metabolic media of fungi PH-S 34 and PH-R 14, isolated from the stems and roots of *P. hispidum*, respectively, showed positive results against the test bacteria *S. aureus*. This Gram-positive bacterium belongs to the family *Staphylococcaceae* and is a multidrug-resistant pathogen that not only causes a diverse array of human diseases, but also is able to survive in potentially dry and stressful environments, such as the human nose, on skin and on inanimate surfaces such as clothing and surfaces (Chaibenjawong and Foster, 2011). The fungal isolate PH-S 34, which produce compounds with activity against this pathogen, proved to be a potential producer of β-caryophyllene (Figure 2) and terpinolene (Figure 3).

The possibility that this endophyte isolate produces secondary metabolites similar to those synthesized by its host, as observed in this study, contributes to understanding the endophyte-host relationship, where the fungus, by producing compounds with antibacterial activity, may help to protect the plant against pathogens (Strobel et al., 2011).

Eight endophytic isolates of *P. hispidum* (PH-L 41, PH-L 44, PH-L 52, PH-L 54, PH-S 23, PH-S 24, PH-R 13 and PH-R 20) produced secondary metabolites that were active in the inhibition of *P. vulgaris*. This pathogen is a Gram-negative bacterium which inhabits the intestinal tract of humans and animals and can also be found in soil, water and fecal matter. Grouped with the *Enterobacteriaceae*, is an opportunistic bacterium of humans, known to cause urinary tract infections (Pelczar et al., 1993). Of the fungal isolates that produce compounds against this pathogen, four were isolated from the leaves, two from the stems and two from the roots. Among the *P. hispidum* leaf isolates which produced metabolites with activity against *P. vulgaris* are PH-L 52 and PH-L 54, possible producers of β-caryophyllene and terpinolene (Figures 2 and 3,

Table 4. Antibacterial activity of metabolic media from endophytic fungi isolated from *P. hispidum* against the bacteria pathogens. Experiments were performed in triplicate.

Endophytic isolates	Pathogenic bacteria			
	S. aureus	***P. vulgaris***	***E. coli***	***S. sonnei***
PH-L 41	-	+	-	-
PH-L 44	-	+	-	-
PH-L 51	-	-	+	-
PH-L 52	-	+	-	+
PH-L 54	-	+	-	+
PH-S 23	-	+	-	-
PH-S 24	-	+	-	+
PH-S 28	-	-	+	-
PH-S 30	-	-	-	+
PH-S 33	-	-	+	-
PH-S 34	+	-	-	+
PH-R 13	-	+	-	-
PH-R 14	+	-	-	+
PH-R 18	-	-	-	+
PH-R 20	-	+	-	-

(+) = Presence of a growth inhibition zone; (-) = no zone of inhibition of bacterial growth.

respectively). Similar results were observed in a study carried out by Cávar et al. (2008) who verified the antimicrobial activity against *S. aureus* of the essential oil of *Satureja montana*, composed of the terpenes geraniol (22.3%), carvacrol (10.6%), terpinen-4-ol (10.3%), caryophyllene oxide (5.2%), spatulenol (3.1%), β-caryophyllene (2.9%), among others. In 2006, Silva et al. reported the isolation of five cadinane sesquiterpene derivatives obtained from *Phomopsis cassiae*, an endophytic fungus isolated from the endemic Brazilian plant *Cassia spectabilis*. The authors found that these sesquiterpenes present remarkable antibacterial and antifungal activities. Therefore, as observed in different studies, terpenes have excellent potential for use as antimicrobial agents, whether produced by the plants themselves or by their endophytes.

There were three endophytic isolates from *P. hispidum* (PH-L 51, PH-S 28 and PH-S 33) that produced metabolites capable of inhibiting the growth of *E. coli*, a Gram-negative bacterium. This has been described as one of the oldest human symbiotic bacteria responsible for serious intestinal infections (Pelczar et al., 1993). Among the isolates that produced compounds with activity against *E. coli,* one was isolated from the leaves and two from the stems of *P. hispidum*. Xing et al. (2011) obtained similar results in a study where they isolated endophytic fungi from *Dendrobium devonianum* and *Dendrobium thyrsiflorum*. The authors verified that more fungi isolated from the stems presented antimicrobial activity when compared with the roots. However, the isolates from roots were more effective against the pathogenic microorganisms. According to Xing et al. (2011), the reason for this contrast is the different environments within the parts of the plant, which influences the distributions and colonization of fungal endophytes.

The *in vitro* antagonist tests revealed that seven of the fungal isolates obtained from *P. hispidum* (PH-L 52, PH-L 54, PH-S 24, PH-S 30, PH-S 34, PH-R 14 and PH-R 18) produced compounds with inhibitory activity against the Gram-negative bacterium *S. sonnei*, the most common causative agent of shigellosis. This disease is spread by fecal-oral route, has high infectivity and is characterized by causing bloody diarrhea, often accompanied by abdominal pain (Pelczar et al., 1993). Knowledge of the epidemiology and molecular mechanisms of antimicrobial resistance in this important pathogen is essential for the implementation of intervention strategies. Ke et al. (2011) verified that in *Shigella* species, antimicrobial resistance is often associated with the presence of integrons that contain resistance gene cassettes. The structures and functions of integrons in *Shigella* species help to describe mechanisms that control integron-mediated events linked to antibiotic resistance. Among the isolates of *P. hispidum* which produced compounds with activity against *S. sonnei*, two were isolated from the leaves,

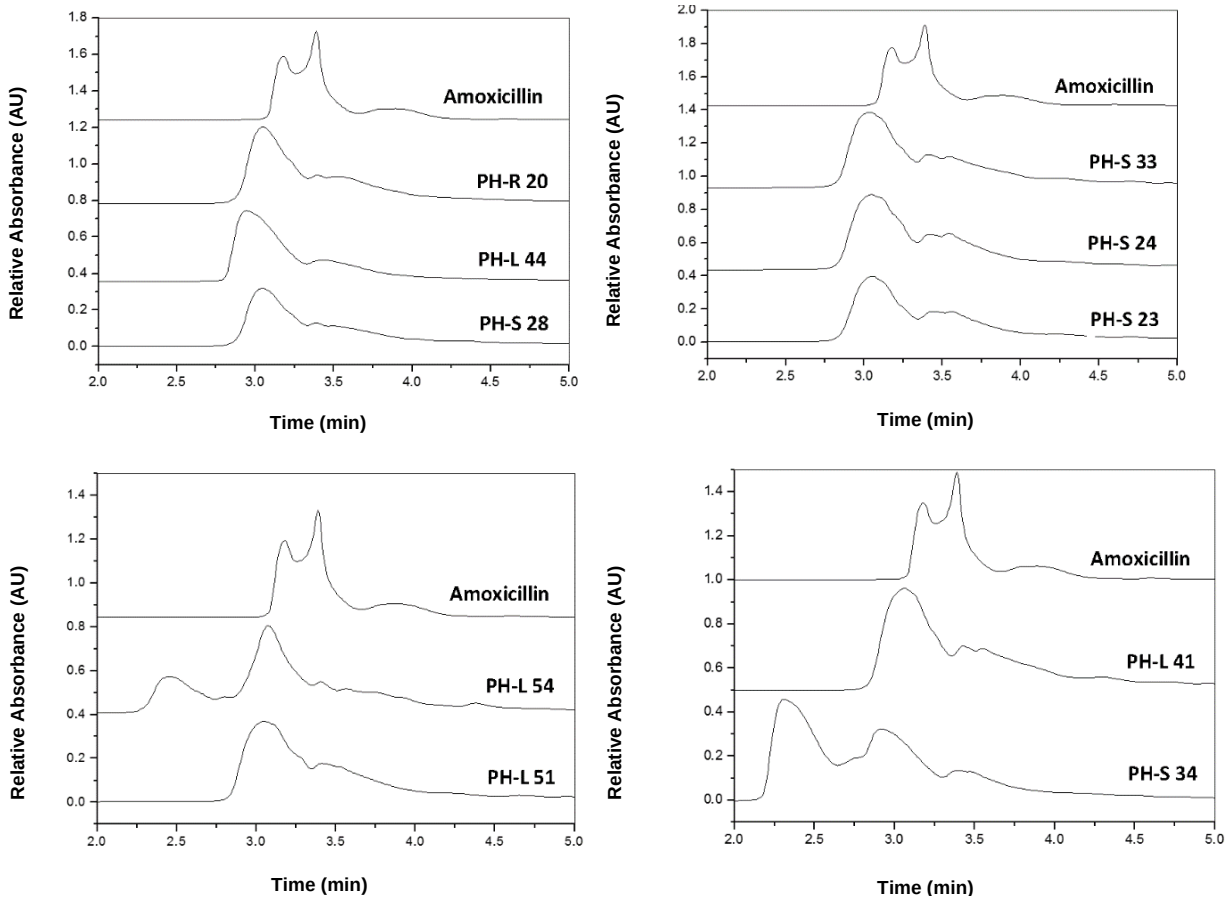

Figure 4. Chromatograms of commercial amoxicillin and metabolic broths of endophytic isolates from *Piper hispidum* that showed antibacterial activity.

three from the stems and two from the roots. Among the isolates obtained from the leaves, as was observed in tests against *P. vulgaris*, are the fungi PH-L 52 and PH-L 54, possible producers of β-caryophyllene and terpinolene (Figures 2 and 3, respectively). Among the isolates from the stem, it can be noted that once again the metabolites of fungus PH-S 34 showed antibacterial activity, as previously observed against the pathogen *S. aureus*. The fact that this endophyte produces compounds with activity against a Gram-positive bacterium and others against Gram-negative bacteria confirms its potential as a producer of secondary metabolites of commercial interest. The Gram-positive and negative bacteria do not differ only with regard to the structure of the cell wall, but also due to the presence of polysaccharides and lipoproteins in Gram-negative bacteria that form a barrier to hydrophobic compounds, which are fundamental aspects for antibiotic action (Mazutti et al., 2008).

From the biological point of view, the production of a particular metabolite can be related to different mechanisms involved in the interaction between a microorganism and its habitat, for example, competition for a niche when a microorganism occupies the same space as the other and competition for nutrients, or it may simply be a product of their metabolism (Li et al., 2007). These results reinforce the idea that the endophytes play a crucial role in the mechanisms of plant protection against pathogens.

Chromatographic profiles of metabolites of endophytic fungi

The 15 extracts of endophytic isolates of *P. hispidum* that provided positive results in the *in vitro* antagonism tests against pathogenic bacteria were analyzed by HPLC to compare their chromatographic profiles with that obtained for a commercial antibiotic (amoxicillin). Ten metabolic broths containing fungal metabolites produced by the isolates PH-L 41, PH-L 44, PH-L 51, PH-L 54, PH-S 23, PH-S 24, PH-S 28, PH-S 33, PH-S 34 and PH-R 20 presented molecules with similar chemical characteristics of the commercial amoxicillin, as can be seen in the chromatograms of Figure 4.

From the analysis of the chromatographic profiles of

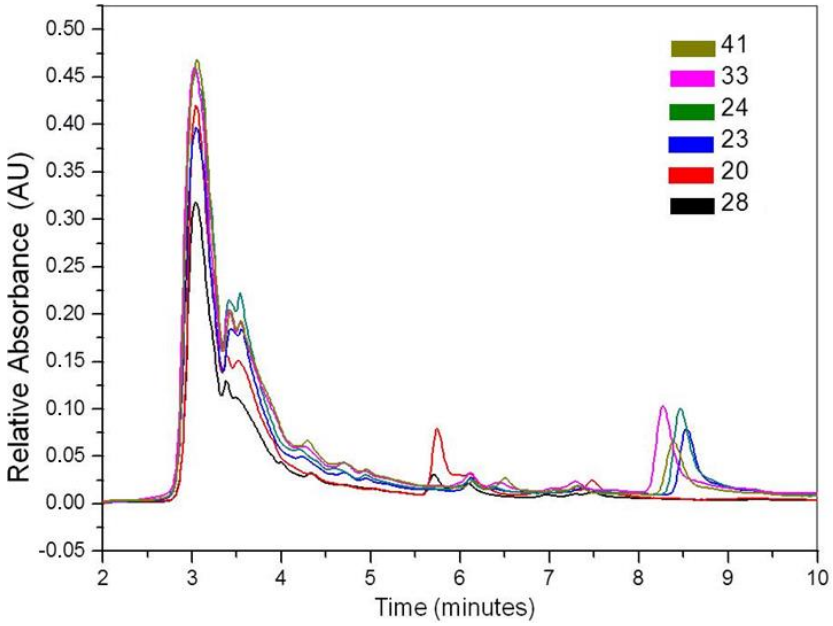

Figure 5. Overlaying chromatograms of the metabolic media of endophytes isolated from *Piper hispidum* that showed antibacterial activity.

Figure 4, it is possible to infer that these endophytic fungi isolated from *P. hispidum* produce compounds that are chemically similar to the commercial antibiotic, since the peaks in the chromatograms obtained appear in the same region. The similarity between the chemical compounds present in fungal metabolites with antibacterial activity and the commercial antibiotic suggests that the mechanism of action of substances produced by these endophytes could be the same as that of amoxicillin, that is, inhibition of the biosynthesis of cell wall proteins (Mikell et al., 2011). It can also be noted in Figure 4 that the chromatographic profile of metabolites from the isolate PH-S 34, which showed activity against Gram-negative and Gram-positive bacteria, indicates the presence of at least five compounds that may act synergistically, or specifically on the bacterial cell wall components.

On analyzing the chromatographic profiles of the metabolic broths of the 15 endophytic isolates from *P. hispidum* that showed antibacterial activity, the production of the same substance was found for the isolates PH-L 41, PH-S 23, PH-S 24, PH-S 28, PH-S 33 and PH-R 20.

The overlaying of the chromatograms of the isolate metabolites is shown in Figure 5. It can be noted that six metabolic broths have the same peak with a retention time of 3.05 min, in the same region of the chromatogram where the peaks were observed for the commercial amoxicillin.

Considering the results for the antibacterial activity of these isolates, and the chemical characteristics being similar to those of amoxicillin, it could be that this molecule with a peak at 3.05 min may be the responsible for the antimicrobial activity of these isolates.

Conclusions

The endophytic fungi isolated from *P. hispidum* exhibit potential for the acquirement of interesting compounds. The HPLC results suggest that three fungal isolates may produce β-caryophyllene and terpinolene. However, it is necessary to obtain confirmation of the production of these molecules using specific techniques such as mass spectrometry and nuclear magnetic resonance.

Antagonist tests confirmed the production of antimicrobial compounds by endophytic fungi from *P. hispidum* with action against the tested pathogen strains (*S. aureus*, *S. sonnei*, *E. coli* and *P. vulgaris*). Comparison of the chromatographic profiles for the fungal metabolites with antimicrobial activity with that for the commercial antibiotic amoxicillin suggests that these fungi produce molecules with chemical structures similar to that of the drug.

Finally, these results indicate a complex interaction between endophytic microbes and their host, with the production of secondary metabolites with proven antibacterial activity, and that the endophyte may be helping the plant to defend itself against different pathogens.

Conflict of Interests

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

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Fibrolytic enzyme production of *Myceliophthora thermophila* M.7.7. using inexpensive carbon sources and mineral nutrients

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This study investigated the effect of inexpensive carbon and nitrogen sources on enzyme production by *Myceliophthora thermophila* M.7.7 in solid-state fermentation. Three kinds of lignocellulosic waste (corn straw, sugarcane bagasse and sugarcane straw) and six nitrogen sources (urea, calcium nitrate, analytical ammonium sulphate, yeast extract, agricultural fertilizer NPK 20-05-20 and fertilizing grade ammonium sulphate) were tested. Some physical-chemical parameters of the fermentation, such as temperature, initial pH and moisture content of the substrate on enzyme production, were evaluated. The maximum activities of xylanase (446.9 U/ml), endoglucanase (94.7 U/ml) and β-glucosidase (2.8 U/ml) were observed in a mixture of corn straw and wheat bran (1:1 w/w) as the carbon source using fertilizer grade ammonium sulphate as the nitrogen source. This production occurred for an incubation period of 96 h, at 40°C, with initial moisture content of 70% and pH 5.0. These results have significant interest since they could be used for the future production of enzymes in a low-cost industrial process.

Key words: *Myceliophthora thermophila*, solid-state fermentation, xylanase, endoglucanase, β-glucosidase.

INTRODUCTION

The hydrolysis of cellulose and hemicellulose present in plant cell walls into glucose and xylose requires the cooperative action of complex enzymes of cellulase and xylanase groups, respectively (Panagiotous et al., 2003; Soni et al., 2010; Danmek et al., 2014). These enzymes have great application potential in several biotechnological processes such as the bioconversion of biomass wastes to fermentable sugars (Fang et al., 2010; Huang et al., 2013).

Enzyme production by filamentous fungi is attractive technologically and has advantages over bacteria and yeasts due to their ability to grow on solid substrates and secrete a higher quantity of extracellular enzyme. These properties make possible the use of agro-industrial residues as substrates in solid state fermentation (SSF) allowing the production of low-cost enzymes (Jecu, 2000;

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Gao et al., 2008; Singh et al., 2009; Longwei et al., 2014). In the fermentation process, it is also important to consider the cost of nitrogen and other macronutrient sources (Su et al., 2011) because, it is very important to find alternatives of using inexpensive mineral nutrients to the culture media. The fermentation parameters such as pH, temperature, moisture, aeration and incubation time influence the expression and secretion of the enzymes significantly affecting the targeted product (Lakshmia et al., 2009).

The heat released by the microbial activity during the fermentation process causes increase of the temperature of the system requiring cooling of the bioreactor. The use of thermophilic fungus in SSF has been very promising since they can adapt to variations in temperature during the process and do not require cooling (Gomes et al., 2009).

This study aimed to evaluate the conditions of xylanase and cellulase production searching an inexpensive fermentative process to make them viable for future industrial application. The strategy used was to cultivate the thermophilic fungus *Myceliophthora thermophila* M.7.7 in SSF using inexpensive agro-industrial waste as carbon sources and commercial agricultural fertilizer as nutrients sources. In addition, the effect of various physico-chemical conditions was evaluated on enzyme production.

MATERIALS AND METHODS

Microorganism and effect of temperature on fungal growth

The strain *M. thermophila* M.7.7 used in this study was isolated from decaying sugarcane bagasse piles and identified by the data derived from BLASTn results using the ITS-rDNA region as a molecular marker which had 99% sequence identity with *M. thermophila* strain ATCC 42464 (Moretti et al., 2012). The culture was maintained on slanted Sabouraud agar (g/L: 40.0 dextrose, 10.0 peptone, 20.0 agar and pH 5.6) under water and mineral oil, at room-temperature (25 ± 2°C) and by spores immersed in 20% glycerol at -80°C.

In order to investigate the performance of the strain at various temperatures, mycelia from pure cultures were spotted on agar plates and incubated at 37, 40, 45, 50 and 55°C. The diameters of the colonies were measured at 12 h intervals. All experiments were performed in replicates of three.

Enzyme production under solid state fermentation (SSF) on different agricultural wastes and mineral nutrients

The effect of lignocellulosic wastes (mixtures of corn straw, sugarcane bagasse or straw with wheat bran, w/w 1:1) on enzyme production was studied according Moretti et al. (2012). The dried substrates were ground to particles of 3 mm and 5 g of the substrate mixture was placed in polypropylene bags (size 12 x 20 cm). The chemical composition of substrates were (% w/w): corn straw was cellulose 28%, hemicellulose 15% and lignin 19%; sugarcane bagasse was cellulose 47%, hemicellulose 16% and lignin 27%; sugarcane straw was cellulose 43%, hemicellulose 15% and lignin 23%.

The nitrogen sources urea, calcium nitrate, ammonium sulphate, yeast extract, agricultural fertilizer NPK (20:05:20 -Heringer) and ammonium sulphate fertilizer grade were tested. Additionally, nutrient solutions were composed of (g/L) 3.0 KH_2PO_4, 0.5 $MgSO_4.7H_2O$, 0.5 $CaCl_2$ and Tween 80 (0.1%), pH 5.0 (modified Mandels and Sternberg, 1976 method) and of a solution formulated with agricultural fertilizer containing (g/L) 10.0 ammonium sulphate, 3.0 mono-ammonium phosphate (MAP) (9% nitrogen and 48% phosphorus) and 2.0 potassium chloride (Heringer), 0.5 $MgSO_4.7H_2O$, 0.5 $CaCl_2$ and Tween 80.

The substrates containing nutrient solution were autoclaved at 121°C for 30 min. The inoculum consisted of 2 x 10^7 spores/g substrate and the final moisture content of the medium was 80%. After inoculation, the material was incubated at 45°C for 10 days and samples were collected at 48 h intervals. Crude enzyme solutions were obtained using suspensions of the fermented material in 100 mL distilled water. The filtrate was centrifuged at 10000 ×*g* for 15 min at 10°C and the supernatant liquid was used as crude enzyme.

Enzyme assays

Xylanase and endoglucanase activities were assayed in reaction mixtures containing 0.1 mL of crude enzyme and 0.9 mL of sodium acetate buffer solution at 0.1 M, pH 5.0 added xylan (birchwood) (10.0 g/L) or carboxymethylcellulose (CMC) (40.0 g/L), which were then incubated at 60°C for 10 min. The free xylose and glucose units produced as a result of xylanase and endoglucanse activity, respectively; react with 1.0 mL of 3-5 dinitrosalicylic acid (DNS) reagent (Miller, 1959). This final reaction forms a colored complex that was measured by spectrophotometer at 540 nm. The enzyme activity was defined in International Units (U), as the amount of enzyme required to release 1 µmol of product per 1 min in the assay conditions.

The β-glucosidase activity was determined according to Leite et al. (2008) in a reaction mixture composed of 0.050 mL of crude enzyme solution, 0.250 mL of sodium acetate buffer (0.1 M; pH 5.0) and 0.250 mL of 4-nitrophenyl-β-D-glucopyranoside (4 mM), (PNPG, Sigma) incubated at 60°C for 10 min. The reaction was stopped by the addition of 2.0 mL of Na_2CO_3 (2 M) and was measured at 410 nm. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per 1 min in the assay conditions.

Effect of incubation temperature, initial pH and moisture on enzyme production

To evaluate the effect of incubation temperature, the two substrate and nutrient supplements which had high enzyme activity in the previous assay were used. The inoculated substrates were incubated at 40, 45, 50°C for 10 days. In all the experiments, the pH and moisture content were maintained as previously described (Gautam et al., 2011).

The effect of initial pH of the media on enzyme production was evaluated at pH 5.0, 5.5, 6.0, using a substrate at 80% of moisture under a temperature that allowed high enzyme activity, for 10 days. The effect of substrate moisture was studied for 60, 70 and 80% using the best substrate and nutrient conditions. For the enzyme extraction, distilled water was added to fermented material at proportions of 1:10 (w/v).

Evaluation of the amount of eluent and use of buffer and surfactant in the enzyme extraction

The fermented material was mixed and divided into five equal parts. Three were used to evaluate the proportion of eluent (1:10, 1:20

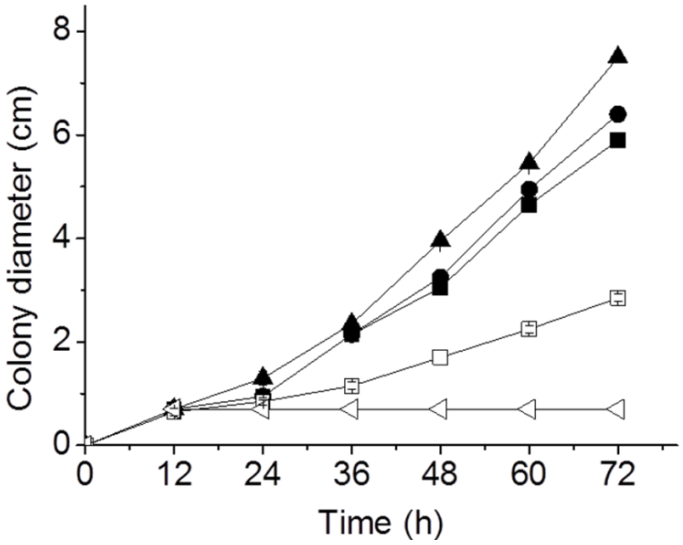

Figure 1. Growth curves of the fungus *Myceliophthora thermophila* M.7.7. in Petri dishes containing solid medium agar Saboroud, at different temperatures, for 72 h. ■ 37°C; ● 40°C; ▲ 45°C; □ 50°C; ◁ 55°C.

and 1:30 w/v) using distilled water and two of them were used to evaluate the type of eluent (Tween 80 at 0.2 mL/L and sodium acetate buffer 0.1M, pH 5.0) at 1:10 (w/v) proportions.

SDS-PAGE analysis and xylanase, endoglucanase and β-glucosidase activity detection by zymogram analysis

Polyacrylamide gel (SDS-PAGE) 10% (w/v) was used for detection of protein bands from crude enzyme solution as described by Laemmli (1970). The molar mass of proteins, under denaturizing conditions, was determined with reference standard proteins (SDS-PAGE Molecular Weight Standards, Broad Range, Bio-Rad from 6.5 to 200 kDa). Protein bands were stained with silver.

For zymogram activities, samples of crude enzymes from *M. thermophila* M.7.7. were mixed in the loading buffer (2% SDS (w/v), 87% glycerol, 0.1 M Tris-HCl buffer pH 8.8 and bromophenol blue). After electrophoretic running, the gels were incubated for 30 min at 60°C in solutions containing xylan or carboxymethylcellulose for xylanase and endoglucanase activity, respectively. After incubation, the gels were stained with 0.1% Congo red solution under gentle shaking for 15 min at 25°C. Subsequently, the gels were immersed in 1 M NaCl solution until the appearance of clear bands on the red background. For better resolution of the bands, 0.1 M HCl was added.

For the β-glucosidase zymogram, after running, the gel was incubated for 10 min at 25°C in 0.2 M acetate buffer, pH 5.0. Subsequently, the gel was incubated for 1 h at 60°C in 0.2 M acetate buffer, pH 5.0 containing 0.1% esculin and 0.03% ferric chloride until the appearance of dark bands when the gel was dipped in 10% glucose solution to stop the reaction.

RESULTS AND DISCUSSION

Effect of temperature on fungal growth

M. thermophila M.7.7 showed maximum growth rate (7.5 cm colony diameter) at 45°C on a solid medium after 72 h of incubation. Colony diameters of 6.4 and 5.9 cm were obtained at 40 and 37°C, respectively. These results confirm the thermophilic profile of the fungus (Figure 1). This evaluation is necessary because the growth response of fungus on a solid medium may differ from that in a liquid medium and provides substantial guidance for the solid state fermentation process. This methodology has been used by other authors showing results consistent (Martin et al., 2010; Silva et al., 2005).

Enzyme production using different agricultural wastes and mineral nutrients in solid state fermentation (SSF)

Corn straw with wheat bran was the best carbon source for xylanase and endoglucanase production by *M. thermophila* M.7.7 (120 and 40 U/mL respectively) while the mixture of sugar cane bagasse and wheat bran allowed the highest β- glucosidase production (Figure 2). Badhan et al. (2007) obtained 62.0 U/ml of xylanase when using cultivated fungus *Myceliophthora* sp. IMI 387099 in sugar cane bagasse but lower amounts of endoglucanase (0.7 U/ml) and β-glucosidase (0.2 U/ml).
In a medium with fertilizer grade ammonium sulphate and analytical ammonium sulphate, maximum xylanase and endoglucanase activity were observed (Figure 2). These values were higher as compared to other studies with fungus of the same genus. According to data from Badhan et al. (2007), the measured activities of xylanase, endoglucanase and β-glucosidase were 90.0, 3.2 and 0.7 U/mL, respectively, when *Myceliophthora* sp. IMI 387099 was cultivated on rice straw with addition of ammonium sulfate (0.3%).

The highest productions of β-glucosidase (4.1 and 3.5 U/mL) were obtained in the media containing yeast extract and urea, respectively. On the other hand, it is clear that the endoglucanase and xylanase production were affected by supplementation with urea (Figure 2). Similar behavior was observed by Kalogeris et al. (2003) using thermophilic *Thermoascus aurantiacus*.

In this study, we can conclude that the corn straw with wheat bran was the best carbon source while ammonium sulfate was the most suitable nitrogen source for endoglucanase and xylanase production. Since there was no difference in enzyme production between the analytical grade and the fertilizer grade of the ammonium sulphate, we opted for the latter as the nitrogen source in the continuity of experiments considering the lower cost of input.

Evaluation of the amount of eluent and use of buffer and surfactant in the enzyme extraction

For enzyme extraction from the fermented solid substrate, three volume and different eluents were used.

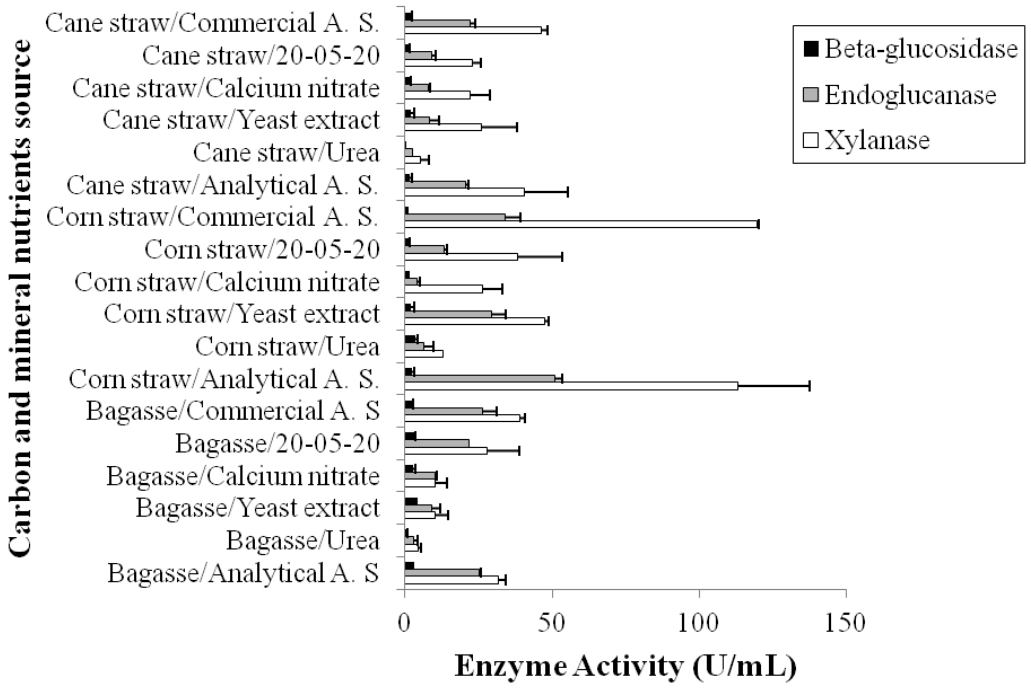

Figure 2. Maximum production of xylanase, endoglucanase and β-glucosidase by *M. thermophila* on different agricultural wastes and mineral nutrient sources. The fermentations were carried out at 45 ºC, pH 5.0, 80% moisture for 240 h. Data are the averages of two assays. Where: analytical A.S. = analytical grade ammonium sulphate, Comercial A.S. = fertilizer grade ammonium sulphate and 20:05:20 = NPK.

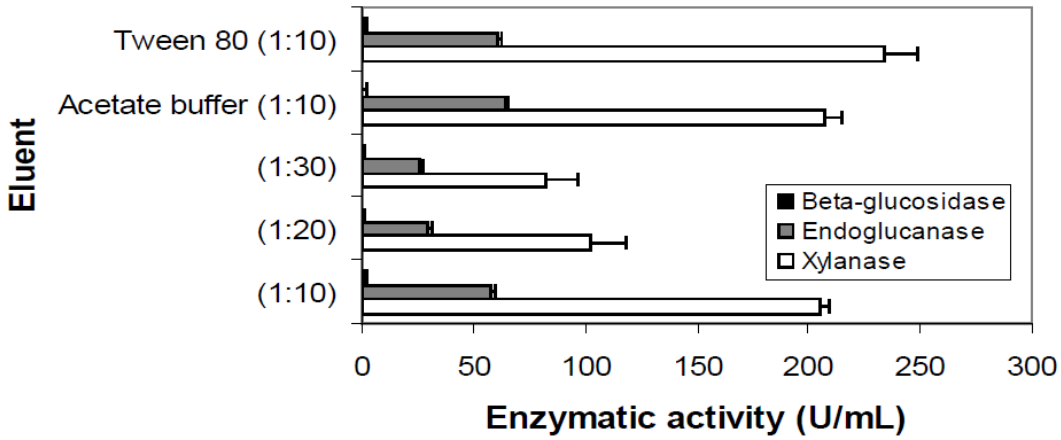

Figure 3. Effect of different eluents and their volume on the enzyme extraction: 1:10, 1:20, 1:30 (1 g of fermented material per distilled water w/v), 1:10 sodium acetate buffer (0.1 M, pH 5.0) and 1:10 Tween 80 (0.2 mL/L). The fermentation was carried out on corn straw and wheat bran (w/w 1:1) and a nutrient solution composed of analytical ammonium sulphate at 45°C, pH 5.0, 80% moisture and incubation for 96 h. Data are the averages of two assays.

According to Figure 3, the ratio of fermented material to water of 1:10 resulted in the highest extraction of enzymes and there were no significant differences among the three eluents, with only slightly higher extraction occurring when tween was used. The specific activities (data not shown) confirm these results. Therefore, it was decided by using water as eluent extraction because the buffer and tween could interfere with the subsequent steps of the study and application of the crude enzymes.

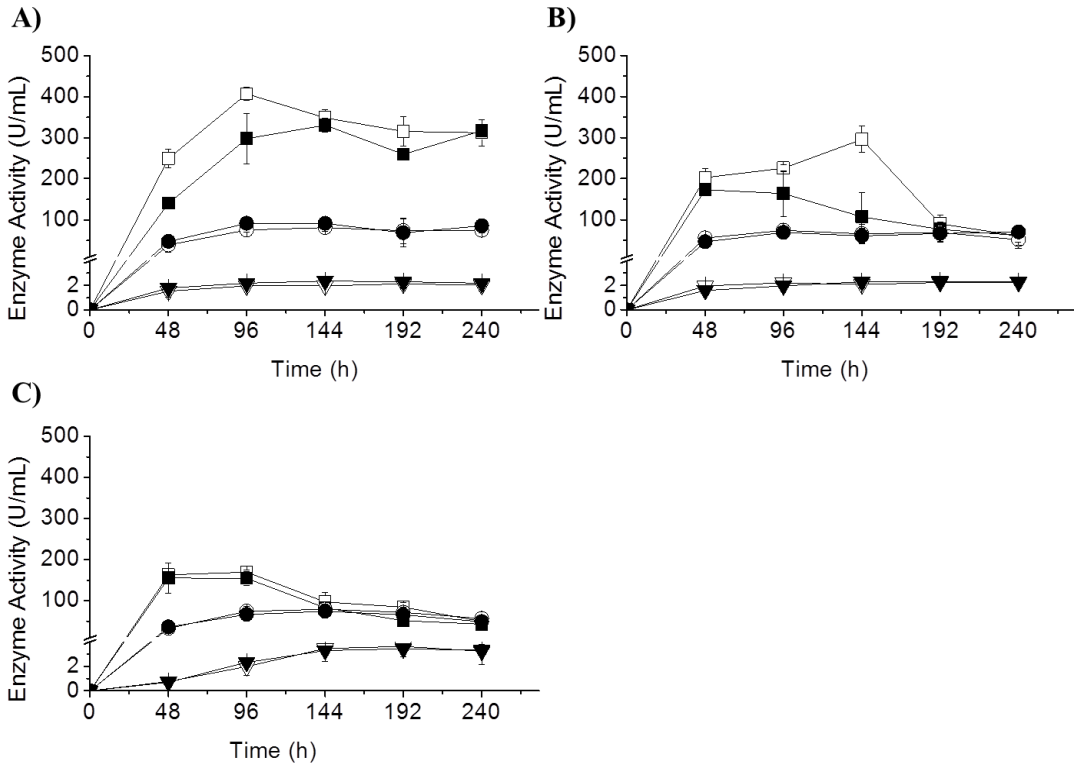

Figure 4. Effect of incubation temperature on SSF. At 40°C (A) 45°C (B) and 50°C (C) on production of xylanase (square), endoglucanase (circle), and β-glucosidase (triangle) by *Myceliophthora thermophila* using a medium consisting of corn straw and wheat bran. The open symbol: analytical grade ammonium sulphate and full symbol: fertilizer grade ammonium sulphate

Effect of incubation temperature on enzyme production

The maximum activity of xylanase (407.0 U/mL) was obtained when *M. thermophila* was grown at 40°C (Figure 4A). When incubated at 45 and 50°C there was a large reduction in the production of xylanase (Figure 4B and C) while the endoglucanase production was little affected by temperature of incubation. The production of β-glucosidase peaked (3.6 U/mL) at 50°C (Figure 4C). These data suggest that temperatures above 40°C could affect the xylanase stability although the growth of fungus was higher at 45°C and also indicated that cellulases were more thermostable than xylanase. Similar results were obtained by Roy et al. (1990) where there was a higher production of β-glucosidase (0.12 U / ml) when *M. thermophila* D14 was cultured at 50°C.

Effect of initial pH and moisture on enzyme production

Figure 5A shows the effect of the pH of the culture medium on the production of cellulases and xylanases. There were no significant differences in the production of enzymes in the extensive pH range tested, with less than 10% variation between the maximum and the minimum activity obtained in the different pH tested, throughout the cultivation period of 240 h. These data corroborate other reported in the literature such as those of Xiong et al. (2004) with xylanase production by *Trichoderma reesei* Rut C-30, those of Shingh et al. (2009) with xylanase from *Coprinellus disseminatus* and those of Sohail et al. (2009) with endoglucanase from *Aspergillus niger* MS82.

The effect of substrate moisture on the production of enzymes by *M. thermophila* is shown in Figure 5B. The maximum xylanase activity (446.9 U/mL) was obtained on the substrate containing 70% moisture after 96 h of cultivation. When the moisture was 60 and 80%, there was a reduction in the xylanase activity (19 and 15%, respectively). The highest endoglucanase (94.7 U/ml) and β-glucosidase (2.8 U/ml) activities were observed after 144 and 240 h of fermentation respectively, with the same profile of xylanase, with a higher production at 70% moisture.

The production of enzymes from *M. thermophila* M.7.7 was quite stable under the effects of a wide range of pH and moisture contents. This characteristic is very interesting for use in industry, since the control of pH and moisture are the most critical parameters to be controlled

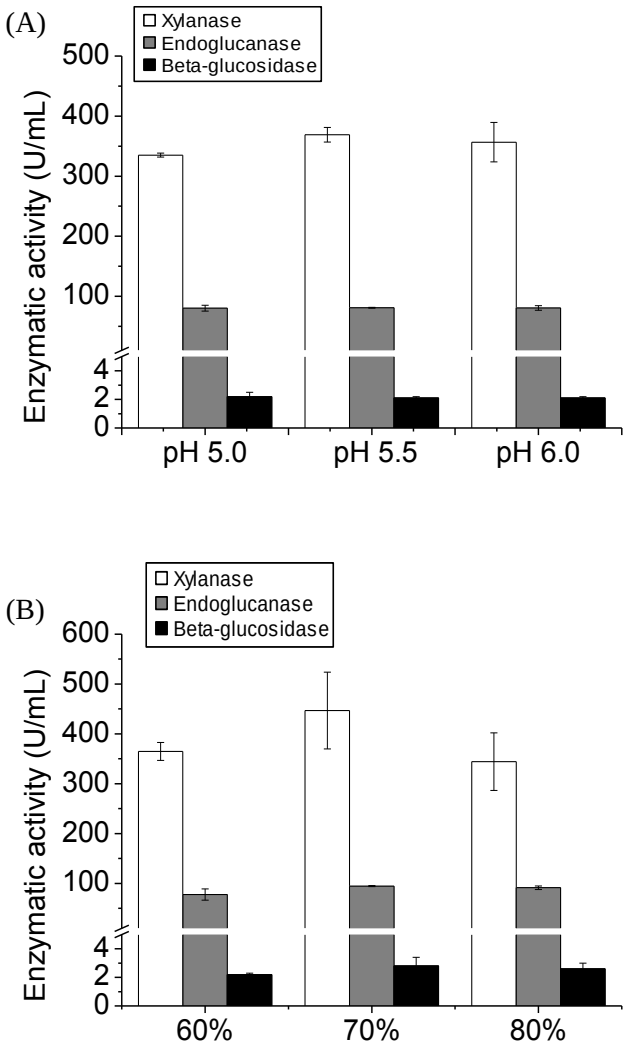

Figure 5. Xylanase, endoglucanase and β-glucosidase production by *M. thermophila* with different initial pH (A) and moisture content (B). The fermentation conditions were carried out on corn straw and wheat bran mixture (w/w 1:1) and a nutrient solution composed of fertilizer grade minerals and ammonium sulphate at 40°C, for 240 h. Data are the averages of two studies.

due to the heterogeneity and the consistency of the solid material normally used as substrate (Lonsane et al., 1985).

SDS-PAGE analysis and xylanase endoglucanase and β-glucosidase activity detection by zymography

The objective of this assay was to verify if the enzyme extract produced by *M. thermophila* M.7.7 on two culture media, using fertilizer grade (1) and analytical grade (2) ammonium sulphate exhibited similar profiles, since ions and metals contained in the first one could inhibit the expression or the activities of the enzymes.

In Figure 6, line B revealed four isoform bands for

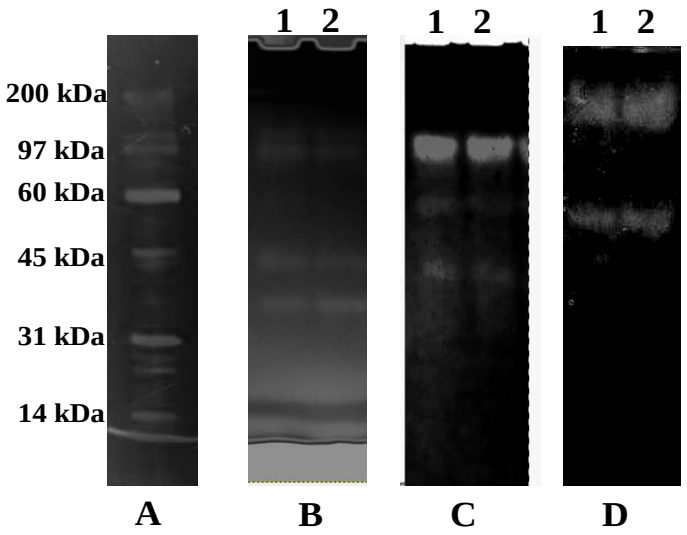

Figure 6. SDS-PAGE and zymogram analysis: (A) molecular weight marker, (B) crude endoglucanase, (C) crude xylanase, (D) crude β-glucosidase. The sample of crude enzymes from *M. thermophila* M.7.7. were obtained under SSF in: corn straw and wheat bran using two different nutrient solutions, fertilizer grade (1) and analytical grade (2) ammonium sulphate.

endoglucanase, corresponding to about 38, 45, 97 and 166 kDa. For xylanase activity (line C), three active isoforms were observed with approximately 43, 60 and 100 kDa. β-Glucosidase appeared in two bands corresponding to 50 and 200 kDa (line D). This similarity in the expression of enzymes in different culture media (1 and 2) shows that nutrient solutions formulated with fertilizer grade ammonium sulphate did not affect the enzymes expression or their activities.

The ability of *M. thermophila* to produce xylanase and endoglucanse in media composed of inexpensive carbon sources and mineral nutrients has commercial interest considering that the substitution of analytical chemical reagents for agricultural fertilizer in the cultivation of fungus reduces the cost of enzyme production. In addition, the fungus used in this study showed great stability in a certain range of temperatures, pH and moisture contents providing a much easier approach to the fermentation process.

Conclusions

In this study, it was established that the best incubation time for enzyme production by the thermophilic fungus *M. thermophila* M.7.7 was 96 h, at which time the maximum activity of xylanase (446.9 U/ml), endoglucanase (77.6 U/ml) and β-glucosidase (2.4 U/ml) was achieved. Corn straw and wheat bran (w/w 1:1) and fertilizer grade minerals can be successfully used as carbon and nitrogen sources. Temperature of 40°C, initial pH 5.0 and moisture content 70% afforded the highest enzyme production.

Conflict of Interests

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

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Full Length Research Paper

Biotechnological potential of actinobacteria isolated from rhizosphere of the medicinal plant, *Ipomoea pes-caprae* (L.) R. Br.

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The aim of the present study was to evaluate the antimicrobial activity and the production of L-asparaginase by actinobacteria isolated from the rhizosphere of the medicinal plant, *Ipomoea pes-caprae* (L.) R. Br., and to conduct a comparative chemical characterization of the methanolic extract from leaves and the most active micro-organism. After isolation, the antimicrobial activity of actinobacteria from rhizosphere were evaluated against *Candida albicans* UFPEDA 1007, *Bacillus subtilis* UFPEDA 86, *Escherichia coli* UFPEDA 224, *Staphylococcus aureus* UFPEDA 02 and clinically isolated *S. aureus* UFPEDA 705. The chemical characterization was conducted by thin layer chromatography for the following groups: triterpenes, steroids, mono- and sesquiterpenes, alkaloids, saponin, coumarin, flavonoids, phenylpropanoids, cinnamic acid derivatives. Finally, a qualitative assay was carried out to evaluate the production of L asparaginase. Among all isolated actinobacteria, the strain *Nocardia* sp. A94 was the most active against Gram positive bacteria, including the clinical isolate (inhibition diameter zone of 23 mm). Additionally, mono/sesquiterpene groups were detected in its methanolic extract, as well in the extract from *I. pes-caprae*. On the other hand, the production of the L-asparaginase was confirmed in 55.17% of tested actinobacteria. The results show the biotechnological potential of actinobacteria from the rhizosphere of *I. pes-caprae* as producers of antimicrobial compounds and L-asparaginase, both activities can be explored for pharmaceutical, cosmetic and food industries.

Key words: Actinobacteria, monoterpene, *Norcadia* sp., rhizosphere, L-asparaginase.

INTRODUCTION

Brazil is home to 20% of the world's biodiversity and is a varied source of bioactive materials in various fields (Azuma, 2002; Silva et al., 2013a). The rhizosphere is a region distinct from the soil, in which there is a diverse and complex interaction between soil, roots and organisms (Ambardar and Vakhlu, 2013). Through this relation, micro-organisms find the substrates necessary for their proliferation and each plant root exudates has a selective effect on its surrounding microbial population (Hartmann et al., 2009). Medicinal plants may thus host a diverse range of micro-organisms that produce bioactive substances, such as antibiotics, anti-tumor agents, immunosuppressants and enzymes (Li et al., 2008, Zhu et al., 2009).

Rhizospheric micro-organisms include actinobacteria, which make up a considerable proportion of the micro-organisms in the soil (10^4 to 10^6 spores per gram of earth) and display a great variety of morphologies (Raju et al., 2010). This group of bacteria is especially important due its capacity to produce bioactive compounds, especially antimicrobials (Bérdy, 2005). Another example is the production of the L-asparaginase enzyme, which converts L-asparagine into L-aspartic acid and ammonia and it has been used as chemotherapy drug to treat acute lymphoblastic leukemia (Dejong, 1972, Sarquis et al., 2004).

The medicinal plant *Ipomoea pes-caprae* (Convolvulaceae) is commonly known as 'morning glory' and it is native to the *restingas* of sandy dunes on the coast of Africa, Asia and Brazil (Wasuwat, 1970; Lorenzi and Matos, 2002). Its use in folk medicine comes from the habits of the Australian aborigines who used to heat the leaves and apply them on boils (Wasuwat, 1970). The microbial environment of the rhizosphere soil habitat of *I. pes-caprae* has not been the subject of much research and it is therefore interesting to attempt characterization and identification of the micro-organisms present in this rhizosphere and their biotechnological applications.

The aim of this present study was to evaluate the antimicrobial activity and the production of L-asparaginase by actinobacteria isolated from the rhizosphere of *I. pes-caprae*, and to conduct a comparative chemical characterization of the methanolic extract from leaves and the most active micro-organism (*Nocardia* sp. A94).

MATERIALS AND METHODS

Sample collection

The rhizosphere and leaves of *I. pes-caprae* were collected in January 2012 on Forte Orange Beach, in the city of Itamaracá (7°48'38''S, 34°50'27''W), in the Brazilian State of Pernambuco. A sample of *I. pes-caprae* (leaves and stem) was deposited in the herbarium of the Agronomic Institute of Pernambuco (Instituto Agronômico de Pernambuco, Brazil; voucher number 85782) and the remaining leaves were washed and subsequently divided up and prepared for the extraction process.

Isolation of Actinobacteria

A sample (10 g) of soil was mixed with 90 mL of 0.9% (w/v) NaCl. The pH (5.6) of this solution was measured and it was agitated mechanically for 20 min and then heated in a water bath to 50°C (Marroni and Germani, 2011). Portions (1 mL) of soil suspensions were transferred to 9 mL of sterile water (diluted 10^{-1}) and subsequently diluted to 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} (King et al., 1954). Shortly thereafter, 200 µL of the dilutions was inoculated in Petri dishes containing 20 mL of culture medium: Tryptone yeast extract - ISP_2 (Pridham and Gottilieb, 1948) and Czapek Dox (CD) Agar medium (sucrose 30.0 g, $NaNO_3$ 3.0 g, K_2HPO_4 1.0 g, $MgSO_4$ x $7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4$ 0.01 g, Agar 15.0 g, H_20 1000 mL, pH 6.6), with added antifungal agent Cyclohexamide 50 µg/mL. The test was carried out in triplicate for each culture medium. One triplicate was prepared with distilled water and in another the medium was enriched with 0.3% NaCl, producing saline Czapek Dox Agar medium. After inoculation, the dishes were incubated in a bacteriological dryer at 37°C for seven days.

Antimicrobial assays

Primary test of antimicrobial activity: "Gelose block"

The test of antimicrobial activity was carried out in accordance with Ichikawa et al. (1971), using micro-organisms from the UFPEDA collection: *Candida albicans* UFPEDA 1007, *Bacillus subtilis* UFPEDA 86 (ATCC 6633), *Escherichia coli* UFPEDA 224 (ATCC 25922), *Staphylococcus aureus* UFPEDA 02 (ATCC 6538) and the clinical isolate *S. aureus* UFPEDA 705.

Semi-solid fermentation of Actinobacteria

The strain which showed the best antimicrobial activity was identified by macro- and micro morphological analysis using the technique described by Shirling and Gottlieb (1966). This strain (*Nocardia* sp. A94) was submitted to semi-solid fermentation using sterile parboiled rice as substrate (Marinho et al., 2005). Twenty small plugs of ISP-2 medium containing aerial mycelium of the *Nocardia* sp. A94 strain were transferred to the twenty-one 500 mL Erlenmeyer flaks containing the solid rice medium (90 g of parboiled rice and 90 mL of distilled water with 0.3% NaCl) and incubated in B.O.D. at 30°C for 21 days (Borges and Pupo, 2006).

Then, cold methanol (150 mL) was added to the cultures of *Nocardia* sp. A94. After 24 h, the extract was filtered in a vacuum. Then a further 100 mL of cold solvent was added to the cultures and they were filtered again. The filtrates were concentrated in a rotary evaporator at 45°C (Borges and Pupo, 2006).

Preparation of plant extract

After drying at room temperature for one week, the sample (leaves) was reduced to small fragments. A total of 5 g of dried leaves was submitted to methanolic extraction for 24 h and filtered with Whatman no.1 paper. The solvent was then removed under reduced pressure in a rotary evaporator at 45°C.

Chemical prospection

The extracts obtained were analyzed using thin layer chromatography (TLC) (Kieselgel 60, 0.2 mm, Merck), with adequate systems and markers for each metabolite group (Harborne; 1998, Wagner and Bladt. 1996) (Table 1).

Qualitative test of production of antitumor L-asparaginase enzyme

The experiment was carried out using the rapid plate test to track

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Table 1. Chromatographic conditions of chemical prospection of methanolic extracts of *Nocardia* sp. A94 and *I. pes-caprae*.

Metabolite	Mobile phase	Marker	Standard	Reference
Triterpenes and steroids	Toluene and ethyl acetate	Lieberman-Burchard	β - sitosterol	Sharma and Dawra, 1991
Monoterpenes and sesquiterpenes	Toluene and ethyl acetate	H_2SO_4, Sulfuric Vanillin	Thymol	Wagner and Bladt, 1996
Alkaloids	Ethyl acetate, formic acid, acetic acid and water	Dragendorff	Pilocarpine	Wagner and Bladt, 1996
Coumarin, flavonoids, phenylpropanoids and cinnamic derivatives	Ethyl acetate, acetic acid, formic acid and water	NEU	Rutin	Wagner and Bladt, 1996

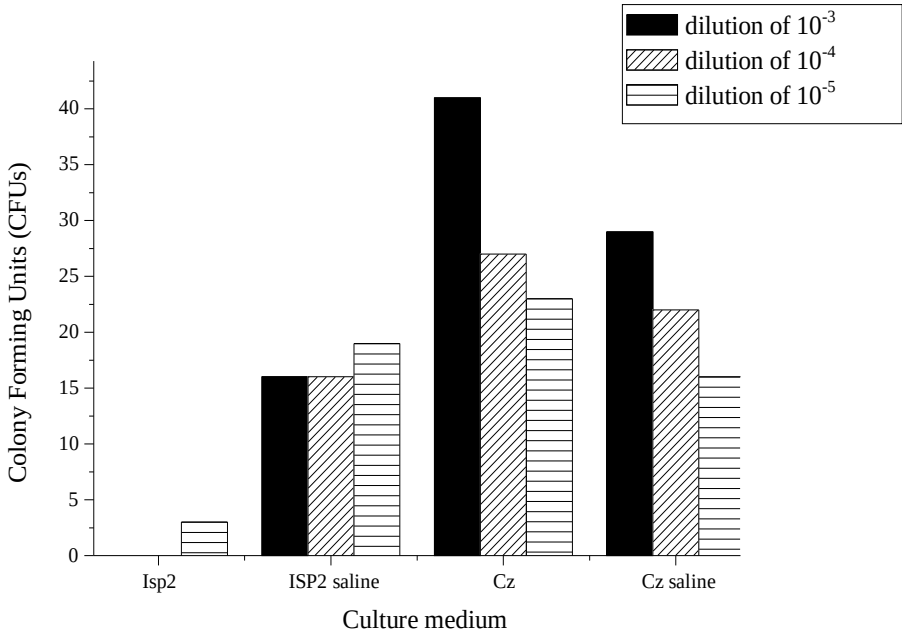

Figure 1. Report of colony forming units of isolated actinobacteria in different culture media ISP_2, ISP_2 saline, Czapek and saline Czapek.

for *in vitro* L-asparaginase production where a modified M-9 medium was prepared, produced with g/L of: $NaHPO_4.2H_2O$ (6.0); KH_2PO_4 (3.0); NaCl (0.5); $CaCl_2.2H_2O$ (0.014); $MgSO_4.7H_2O$ (0.5); glucose (2.0); L-asparagine (5.0); 0.25% ethanolic solution of phenol red (0.09) and agar (17). Phenol red coloring was used in the culture medium as an indicator of changes in pH caused by cleavage of L-asparagine into aspartic acid, changing the color from orange to pink (Gulati et al., 1997). The actinobacteria were incubated at 30°C in a Biological Oxygen Demand (BOD) dryer for seven days.

RESULTS AND DISCUSSION

Isolation and antimicrobial activity of Actinobacteria strains

It was possible to quantify 212 colony forming units (CFUs) in the rhizosphere of *I. pes-caprae* at dilutions of 10^{-3}, 10^{-4} and 10^{-5}. Of these, 81 were isolated. Czapek Dox Agar culture medium was the best for isolating colonies at all dilutions (Figure 1), with the highest number of colonies (41 at a dilution of 10^{-3} = 41 x 10^3) as shown in Figure 1. Of these 81 actinobacteria, only 29 grew with aerial mycelium after isolation.

The rhizosphere is a region of the soil that is influenced by the roots of plants and is characterized by a high level of microbial activity (Hartmann et al., 2009; Lin et al., 2010). The presence of actinobacteria has also been reported for rhizosphere of some medicinal plants such as *Rumex patientia* (Qi et al., 2012), *Crocus sativus* (Hainan and Vakhlu, 2012) and some Bangladesh medicinal plants (Ara et al., 2013). For example, a huge population of actinobacteria (168 x 10^3 CFU per gram of soil) where found in these Bangladesh medicinal plants. Similarity, a total of 400 strains of actinobacteria was

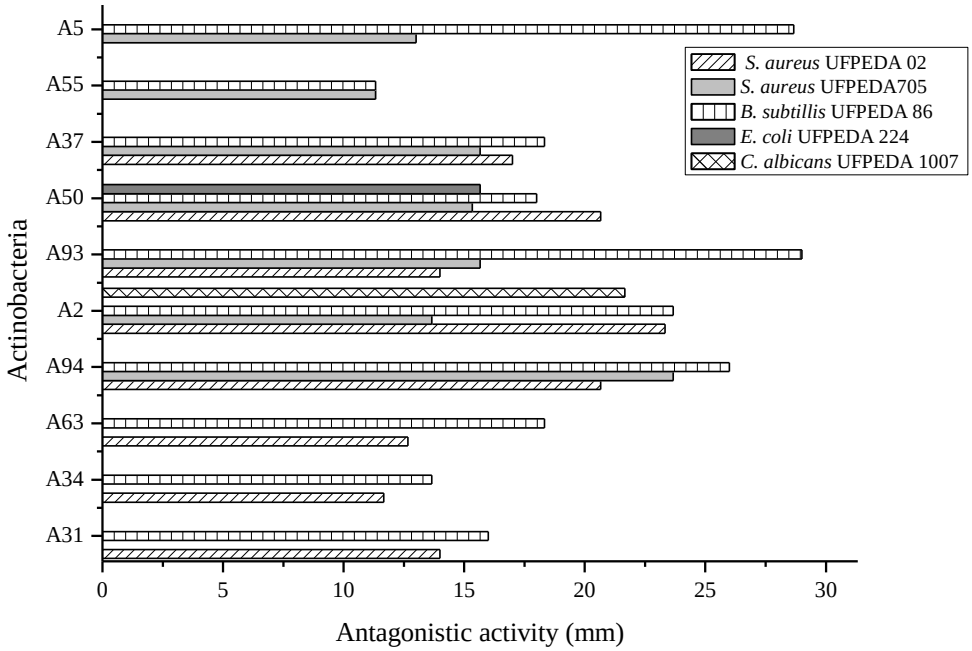

Figure 2. Antimicrobial activity of actinobacteria from rhizosfere of *I. pes-caprae* (Forte Orange, Itamaracá-PE, Brazil).

isolated from 12 soil samples of medicinal plant collected from tropical botanical garden in Danzhou city (Xiaolong et al., 2012).

Although these actinobacteria are from the habitat of the rhizosphere of *I. pes-caprae*, a plant found in dunes on the Brazilian coast (Wasuwat, 1970; Lorenzi and Matos, 2002), the highest number of isolates were found in a non-saline culture medium, suggesting that these actinobacteria are halotolerant (Carro et al., 2013). After purification of strains of actinobacteria from the rhizosphere, only 29 grew with a sufficient number of secondary hyphae to have antimicrobial properties. Sixty-two percent presented activity against at least one test micro-organism. The A2, A50 and A94 actinobacteria, identified as species of the *Nocardia* genus, presented antibacterial activity with inhibition diameter zone (IDZ) of 23.33, 20.66 and 20.66 mm for *S. aureus* UFPEDA 02; 13.66, 23.66 and 15.33 mm for *S. aureus* UFPEDA 705; and 23.66, 18.00 and 26.00 mm against *B. subtilis* UFPEDA 86, as shown in Figure 2. The A2 strain was the only one that presented antifungal activity against *C. albicans* (21.66 mm).

Similar antimicrobial activity was reported in a study of *Streptomyces* (BT-408) isolated from the sediment of the Bay of Bengal in the Indian Ocean, aiming to evaluate its activity against methicillin-resistant *S. aureus* (MRSA). The authors reported that this actinobacteria presented a IDZ of 20 mm (Sujatha et al., 2005) and a study of actinobacteria isolated from the Visakhapatnam region found that, after fermentation and isolation of compounds, the B01 fraction presented moderate activity against *E. coli*, *B. subtilis*, *S. aureus*, *P. aeroginosa* and *B. cereus* (Rao et al., 2012).

The search for secondary metabolites that are active against species of the genus *Staphylococcus* is important, especially to combat *S. aureus* (Da Silva et al., 2013a, b), owing to its high incidence in both hospital and community acquired infections, linked to its patterns of resistance and factor virulence expression (Gould et al., 2012). This problem is compounded when faced with multi-resistant strains of the *S. aureus* (Davis et al., 2013).

Identification and chemical characterization of methanolic extract of strain A-94

Amongst all tested bacteria (29), the strain A-94 showed the best antimicrobial activity against Gram-positive pathogens, especially *S. aureus*; therefore, the strain A-94 was selected to be partially identified and chemical characterized. The microculture technique identified the A-94 strain at the genus level as *Nocardia*. The strain A-94 exhibited well-developed vegetative hyphae with irregular branches penetrating the agar and bearing white aerial sparse hyphae. At a late stage of their growth, the filaments fragment into rod-shaped elements characteristic of the family Nocardiaceae and the genus *Nocardia* (Hoshino et al., 2004).

A chemical characterization was conducted for the MeOH extract of the biomass of *Nocardia* sp., and the MeOH exctract of *I. pes-caprae*. Mono- and sesquiterpenes were found both in the extract of the micro-organism and that of the plant. The other

Table 2. Evaluation of chemical characterization of MeOH extracts of *Nocardia* sp. when compared with MeOH extracts of *I. pes-caprae*.

Metabolite	*Nocardia* sp. extract	Plant extract
Triterpenes and steroids	Negative	Negative
Monoterpenes and sesquiterpenes	Positive	Positive
Alkaloids	Negative	Negative
Coumarin, flavonoids, phenylpropanoids e and cinnamic derivatives	Negative	Positive (flavonoid)

Table 3. Evaluation of production of L-asparaginase by actinobacteria isolated from the rhizosphere of *I. pes-caprae* oriunda from Forte Orange Beach, Itamaracá - PE.

Actinobacteria	Qualitative Test	Actinobacteria	Qualitative Test
A31	+++	A208	+++
A34	+++	A119	ND
A63	+++	A180	++
*A94	+++	A101	++
A2	+++	A222	+++
A93	ND	A210	ND
A50	+++	A207	++
A37	+++	A209	ND
A55	+++	A196	+++
A5	+++	A231	ND
A65	ND	A114	+++
A122	+++	A5.5	+++
A202	+++	A201	++
A120	++	A118	+
UFPEDA 224	+++	UFPEDA 224	+++

ND = Not degraded; + = low level of degradation of L-asparagine in 48 h; ++ = medium level of degradation of L -asparagine in 48 h; +++ = high level of degradation of L-asparagine in 48 h. **Nocardia* sp. A9.

secondary metabolites under study were not found, as shown in Table 2. The presence of sesquiterpenes and other secondary metabolites in the extract of *I. pes-caprae* has previously been reported (Pongprayoon, 1991, 1992). Both monoterpenes and sesquiterpenes are associated with antibacterial, antifungal and antitumor activity (Schwab, Fuchs and Huang, 2013). These compounds are used as components in fragrances, cosmetics, cleaning products, disinfectants, food additives and medicines, owing to their pleasant smell and antimicrobial properties (Schwab, Fuchs and Huang, 2013). Although chemical prospection is generally carried out with extracts from different parts of the plant, such as leaves and roots, it is important to bear in mind that, in the rhizosphere, there are inter- and intra-species relations between micro-organisms and the environment, which causes it to produce similar compounds (Kent and Triplett, 2002).

Qualitative test of production of L-asparaginase enzyme

Additionally, a qualitative evaluation of the L-asparaginase production was performed with all strains isolated from rhizosphere of *I. pes-caprae*. The result was recorded as positive according to a change of color in the M-9 medium. The coloration is the result of degradation of the asparagine in the growth medium caused by the breakdown of L-asparagine into L-aspartic acid and ammonia, which changes the color from orange to pink (Amena et al., 2010). In the screening, 55.17% of strains tested presented an area of degradation of asparagine similar to the positive control, *E. coli* UFPEDA 224, and 20.68 % presented an area of degradation with weaker coloration in comparison with the positive control (Table 3).

L-asparaginase is an enzyme that possesses antineoplastic properties against acute lymphoblastic leukemia and can be produced by a variety of micro-organisms, such as bacteria, actinobacteria and fungi (Dejong, 1972; Moura et al., 2004). *E. coli* and *Erwinia chrysanthemi* are two well-known producers of L-asparaginase, nevertheless, there is a need to search for micro-organisms able to produce high amounts of L-asparaginase, with more therapeutic efficacy and, if

possible, with fewer side effects (Nagarethinam et al., 2012). It is established that preparations from each kind of species differ on their pharmacological properties (therapeutic efficacy, bioavailability and induction of side effects) structural characteristics and physico-chemical kinetics (Labrou et al., 2010). In fact, different habitats have been explored to obtain these micro-organisms as soil (Shukla et al., 2014), marine (Basha et al., 2009), leaves (endophytic) (Chow and Ting, 2014) and rhizosphere (Khamna et al., 2009). For example, actinobacteria strains isolated from 16 samples of rhizosphere of medicinal plants from Thailand and, of 445 isolated, 30 were found to produce this enzyme (Khamna et al., 2009).

Conclusion

The present study suggests that the micro-organisms present in the rhizosphere of *I. pes-caprae* possess biotechnological properties since they are producers of antimicrobial compounds and L-asparaginase. Both activities are of interest to different industries such as pharmaceutical, food, cosmetic and cleaning industries. Further studies on the molecular characterization of each active isolate and purification of the bioactive compounds are in progress.

Conflict of Interests

The authors have not declared any conflict of interests.

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