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Isolation and Characterization of Antifungal Peptides Produced by *Bacillus amyloliquefaciens* LBM5006

Lisianne Brittes Benitez, Renata Voltolini Velho, Marcia Pagno Lisboa, Luis Fernando da Costa Medina, and Adriano Brandelli*

Laboratório de Bioquímica e Microbiologia Aplicada, Departamento de Ciência de Alimentos, ICTA, Universidade Federal do Rio Grande do Sul, 91501-970 Porto Alegre, Brazil

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Bacillus amyloliquefaciens **LBM 5006 produces antagonistic activity against pathogenic bacteria and phytopathogenic fungi, including *Aspergillus* spp., *Fusarium* spp., and *Bipolaris sorokiniana*. PCR analysis revealed the presence of *ituD*, but not *sfp* genes, coding for iturin and surfactin, respectively. The antimicrobial substance produced by this strain was isolated by ammonium sulfate precipitation, gel filtration chromatography and 1-butanol extraction. The ultraviolet spectrum was typical of a polypeptide and the infrared spectrum indicates the presence of peptide bonds and acyl group(s). The antimicrobial substance was resistant to proteolytic enzymes and heat treatment, and was reactive with ninhydrin. Mass spectroscopy analysis indicated that *B. amyloliquefaciens* LBM 5006 produces two antimicrobial peptides, with main peaks at *m*/*z* 1,058 Da and 1,464 Da, corresponding to iturin-like and fengycin-like peptides, respectively. *B. amyloliquefaciens* LBM 5006 showed significant activity against phytopatogenic fungi, showing potential for use as a biocontrol agent or production of antifungal preparations.**

Keywords**:** *B. amyloliquefaciens*, bacteriocin, antifungal activity, lipopeptide

Plant diseases caused by viruses, bacteria and fungi affect crops, and are responsible for significant losses or decrease the quality and safety of agricultural products. Their control relies mainly on chemical pesticides (Agrios, 2005). Because of their rapid bactericidal and/or fungicidal activity over a broad spectrum and the low propensity of microorganisms to develop resistance against them, the phenomenon of microbial antagonism mediated by peptides has gained more attention in recent years. This antimicrobial arsenal has been isolated from almost all species - microorganisms, plants, insects and other invertebrates, fish, amphibians, birds and mammals - and it has been proposed that they represent components of their immediate nonspecific defense against invading bacteria, enveloped viruses, and fungi (Hancock and Diamond, 2000; Ajesh and Sreejith, 2009).

Antimicrobial peptides are widespread produced among diverse bacteria (Riley and Wertz, 2002). Although those produced by lactic acid bacteria have been extensively studied because their potential use in food industry (O'Sullivan *et al*., 2002), interesting antimicrobial peptides are produced by other classes of bacteria such as *Bacillus* spp. (Motta *et al*., 2007) and coryneform bacteria (Motta and Brandelli, 2009). The genus *Bacillus* includes varied industrially important species and has a historical of safe use. The production of antimicrobial peptides has also been described for this genus, and subtilin, subtilosin A, iturin A, bacilysin, rhizocticin, and surfactin are some of these substances (Le Marrec *et al*., 2000; Pabel *et al*., 2003; Romero *et al*., 2004). Many strains of *Bacillus amyloliquefaciens* are known to suppress fungal and bacterial growth *in vitro* by the production of several antimicrobial compounds (Yoshida *et al*., 2001; Caldeira *et al*., 2008; Sutyak *et al*., 2008).

The antifungal activity of *Bacillus subtilis* and *B. amyloliquefaciens* can be attributed, to a certain extent, to production of iturins (Tsuge *et al*., 2001). Iturins are heptapeptides linked to a β-amino fatty acid and iturin A shows a strong antibiotic activity with a broad antifungal spectrum, making it an ideal potential biological control agent with the aim of reducing the use of chemical pesticides in agriculture (Maget-Dana and Peypoux, 1994). Gene clusters involved in iturin synthesis have been investigated (Tsuge *et al*., 2001; Yao *et al*., 2003). The iturin A operon spans a region more than 38 kb long and is composed of four open reading frames: *ituD*, *ituA*, *ituB*, and *ituC*. The *ituD* gene encodes a putative malonyl coenzyme A transacylase, whose disruption results in a specific deficiency in iturin A production (Tsuge *et al*., 2001). Thus, the *ituD* gene play leading role in the production of iturin.

The environment is the main source for new microorganisms with potential industrial or commercial value. The Brazilian Atlantic forest is an ecosystem with enormous biological diversity. The forest has been reduced to less than 10% of its original size, but many efforts are now being made to conserve the remaining biodiversity of this region (Matos and Bovi, 2002). However, there are only a few studies on the microbial diversity of this region (Nascimento *et al*., 2003; Evans *et al*., 2004). The aim of this work was the purification and identification of antimicrobial peptides produced by *Bacillus amyloliquefaciens* LBM 5006 isolated from soil of Atlantic Forest.

* For correspondence. E-mail: abrand@ufrgs.br; Fax: +5551-3308-7048

Table 1. Antifungal activity of *B. amyloliquefaciens* LBM 5006

Indicator fungi	Antifungal activity[a]		
	Direct antagonism	Crude supernatant	Purified
Aspergillus niger ATCC 16404	7.0±0.8	10.5±0.7	10.0±0
Aspergillus phoencis	13.5±1.7	0.0	16.5±0.7
Aspergillus flavus	13.5±2.3	13.5±2.1	14.0±2.8
Apiosordaria sp. [b]	16.0±0.8	13.5±2.1	13.0±1.4
Bipolaris sorokiniana	22.2±1.7	13.5±2.1	15.0±1.4
Cercosporina sojina	21.0±1.1	21.0±1.4	17.5±3.5
Diplodia sp.	6.0±0.8	15.5±0.7	15.0±1.4
Fusarium oxysporum f. licopersici	20.5±2.9	0.0	15.5±0.7
Fusarium graminearum	24.5±1.3	0.0	12.5±0.7
Promopsis sp.	0.0	ND	ND
Rhizoctonia sp.	0.0	ND	ND
Verticillium albatrum	0.0	ND	ND

[a] Values are the Means±SEM of 3 independent determinations of the inhibition zone (mm). ND, not determined.
[b] *Apiosordaria* sp., *Diplodia* sp., *Promopsis* sp., *C. sojina*, *F. graminearum*, and *V. albatrum* were gently given by M.Sc. Nelson Neto, UNICRUZ (Universidade de Cruz Alta, Brazil); other strains were from our culture collection.

Materials and Methods

Microorganisms

The producer strain *B. amyloliquefaciens* LBM 5006, was isolated and characterized as described elsewhere (Lisboa *et al*., 2006). Other bacterial strains were *Bacillus subtilis* ATCC 19659 and *Listeria monocytogenes* ATCC 7644. The strains were maintained at -21°C as frozen stocks in brain heart infusion broth (BHI; Oxoid, UK) containing 20% (v/v) glycerol and propagated twice in the same medium at 37°C before use. Indicator fungal strains used in the antagonist assays are listed in Table 1. Fungi were maintained on potato dextrose agar (PDA; Biobras, Brazil) slants at 4°C.

Fungal suspension and antifungal assays

Strain LBM 5006 was tested for its ability to inhibit the growth of various fungal plant pathogens (Table 1). Fungal isolates were inoculated on plates with PDA for 7 days and 12 h photoperiod. Aliquots of 3 ml of saline solution were poured on each of the colonies and the spores removed with the aid of a loop of Drigalski. The suspension was collected and transferred to a sterile tube. The number of conidia was performed in a Neubauer chamber and the concentration adjusted for 5×10^3 conidia/ml. One mililiter of this suspension was added to 99 ml of sterile PDA at 45°C (Leifert *et al*., 1995) translated and homogenized. The strain *B. amyloliquefaciens* LBM 5006 was inoculated with the aid of a needle in 4 points for plate previously prepared with the fungal inoculum. The inhibition zones were measured after five days of incubation at 25±2°C and 12 h photoperiod. The isolates that showed inhibition zones were selected to additional tests with the extracts obtained from *B. amyloliquefaciens* LBM 5006.

The assay for antifungal activity with culture supernatants and purified substance was carried out on PDA plates (Ye *et al*., 1999). Around the central disk (0.5 cm in diameter) sterile paper disks (0.5 cm) were placed at 1 cm from it. Aliquots (10 µl, 3200 AU/ml) of the test samples were added to the disks. The plates were incubated at 30°C for 48 h until mycelia growth from the central disk had enveloped peripheral disks containing the negative control (phosphate buffered saline) and had formed a crescent-shaped zone of inhibition around disks containing samples with antifungal activity.

PCR analysis

DNA was extracted from overnight cultures of *B. amyloliquefacies* LBM 5006 and *B. subtilis* ATCC 19659 using the Promega Wizard SV Genomic DNA kit (Promega Corp., USA). Specific primers for the functional genes of the bacteriocins iturin A and surfactin are listed in Table 2. PCR was conducted using an Mastercycler® Personal Eppendorf (Eppendorf AG, Germany) under the following parameters: denaturation for 1 min at 94°C, annealing for 1 min at 50°C, and elongation for 1.5 min at 72°C for a total of 30 cycles for iturin A and denaturation for 1 min at 94°C, annealing for 30 sec at 46°C, and elongation for 1 min at 72°C for a total of 25 cycles for surfactin.

Sequencing methods

PCRs products were sequenced in the ACTGene Laboratory (Centro de Biotecnologia, UFRGS, Brazil) using the automatic sequencer ABI-PRISM 3100 Genetic Analyzer armed with 50 cm capillaries and POP6 polymer (Applied Biosystems). Sequencing data were collected using the software Data Collection v1.0.1 (Applied Biosystems). The BLAST algorithm was used to retrieve for homologous sequences in GenBank (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]) using the software CLUSTAL W, version 1.8 (Thompson *et al*., 1994). The 16S rDNA sequence obtained for *B. amyloliquefaciens* LBM 5006 (1,499 bp) has been submitted to GenBank under the accession number GU368920.

Production, isolation, and purification of antimicrobial substance

For the production of antimicrobial substance, the strain LBM 5006 was grown in 200 ml of BHI medium at 37°C in a rotary shaker at 125 cycles/min for 48 h. After cultivation the cells were harvested by centrifugation at 10,000×g for 15 min, the culture supernatant was sterilized by filtration with 0.22 µm membranes (Millipore, USA). The filtrate was precipitated with ammonium sulfate at 20% (w/v) saturation and the resulting pellet was dissolved in 10 mM phosphate buffer pH 6.0. This solution was further purified by gel filtration chromategraphy using Sephadex G-100 eluted with this same buffer and the fractions showing antimicrobial activity were pooled and freeze-dried. Fractions were also monitored for A280 nm using a spectrophoto-

Table 2. Primers for the functional genes of iturin A and surfactin

Bacteriocin	Primer	Sequence (5´→3´)	Reference
Iturin A	*ituD-f*	ATGAACAATCTTGCCTTTTTA	Hsieh *et al.* (2008)
	ituD-r	TTATTTTAAAATCCGCAATT	
Surfactin	*sfp-f*	ATGAAGATTTACGGAATTTA	Hsieh *et al.* (2004)
	sfp-r	TTATAAAAGCTCTTCGTACG	

Table 3. Purification of antimicrobial activity of *B. amyloliquefaciens* LBM 5006

Purification step	Total protein (mg)	Total activity (AU)	Specific activity (AU/mg)	Purification fold	Yield (%)
Crude supernatant	320	80,000	250	1.0	100
$(NH_4)_2SO_4$ precipitation	3.7	12,800	3459	13.8	16
Sephadex G-100 chromatography	3.6	12,800	3555	14.2	16
1-Butanol extraction	0.7	3,200	4571	18.3	4

meter (UV-mini 1240, Shimadzu, Japan). This material was dissolved in 10 mM phosphate buffer pH 6.0 and then extracted twice with l-butanol. The organic phases were combined and evaporated under reduced pressure. The resulting powder was suspended in 10 mM phosphate buffer pH 6.0. During purification the antimicrobial activity (AU/ml) was quantified as described elsewhere using *L. monocytogenes* ATCC 7644 as indicator organism (Motta and Brandelli, 2002), and antifungal activity was confirmed against *F. oxysporum*.

Analytical methods

Soluble protein concentration was determined by the Folin phenol reagent method (Lowry *et al*., 1951). Concentration of free amino groups was determined by the ninhydrin method (Moore and Stein, 1957).

Effects of enzymes and heat on antimicrobial activity

Samples of the antimicrobial substance were treated at 37°C for 1 h with 2 mg/ml final concentration of the following enzymes: papain, trypsin, proteinase K, and pronase E. Samples were then boiled for 2 min to inactivate the enzyme. To analyze thermal stability, samples were exposed to temperatures ranging 25 to 90°C for 30 min, and 121°C/141 kPa for 15 min. The pH stability was determined by adding samples of the antimicrobial substance to McIlvaine buffered solutions (pH 2 to 8) or 0.1 M bicarbonate buffer (pH 9 to 11) and assaying the activity after 2 h at 37°C. After the treatments, the samples were tested for antimicrobial activity against *L. monocytogene*s ATCC 7644 and *F. oxysporum*.

Spectroscopy

The ultraviolet absorbance spectrum was recorded using a Shimadzu UV1601PC double bean spectrophotometer (Tokyo, Japan), from 190 to 600 nm at 0.5 nm resolution. The infrared spectrum was measured as a potassium bromide pellet. Four scans of the sample were taken using a Mattson 3020 FITR spectrophotometer (Madison, USA).

Mass spectrometry analysis was performed after the peptides were eluted from a C18 chromatographic resin (Vydac, USA). The column was eluted with 80% acetonitrile 0.046% TFA and concentrated in a vacuum centrifuge (SpeedVac SC100, Savant, USA). The sample was analyzed in a MALDI-TOF mass spectrometer (Ettan MALDI-TOF ProSystem, Amersham Biosciences, Sweden) operating in reflecton mode and using a matrix of α-ciano-4-hydroxycinnamic acid.

Results

Antifungal activity of *B. amyloliquefaciens* LBM 5006

Spectrum of antifungal activity of LBM 5006 strain was determined based on the degree of growth inhibition of phytopathogenic fungi by direct antagonism on agar plates. In this experiment, LBM 5006 showed inhibition against nine of the twelve tested fungal plant pathogens. There was no activity against *Promopsis*, *Rhizoctonia*, and *Verticillium* sp. (Table 1).

The antifungal activity of culture supernatants of *B. amyloliquefaciens* LBM 5006 was also tested against the indicator fungi. The inhibitory activity of the filter-sterilized crude supernatant was observed on 60% of the strains tested (Table 1).

Identification of genes related to antimicrobial peptides

PCR analysis of the *B. amyloliquefaciens* LBM 5006 showed that the strain exhibited potential for the functional gene-encoding malonyl CoA transacylase (*ituD*) but not for to putative transcription terminator gene (*sfp*) (Fig. 1). The DNA sequence of the amplified PCR product was determined. Sequences of 1203 bp fragments showed elevated similarity (minimum identity 98%) with the gene-encoding malonyl CoA transacylase (*ituD*), and only point mutations were observed.

Isolation and characterization of antimicrobial substance

The antimicrobial activity produced by *B. amyloliquefaciens* LBM 5006 was purified from the culture supernatant. The purification steps and the recovery values are summarized in Table 3. The final specific activity of the antimicrobial substance was increased approximately 18-fold compared to that in the culture supernatant and the recovery was 4%. The purified substance inhibited growth of all tested fungal plant pathogens (Table 1), as illustrated in Fig. 2 for *F. oxysporum*.

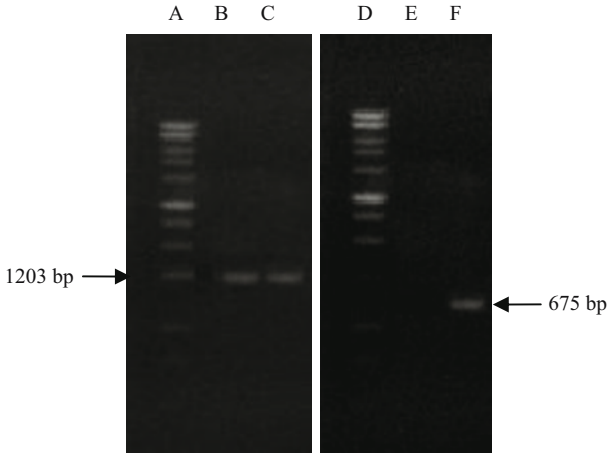

Fig. 1. PCR product profiles of representative samples tested. Lanes: A and D, Molecular weight marker; B, gene *ituD* for *B. amyloliquefaciens* LBM 5006; C, *B. subtilis* ATCC 19659; E, gene *sfp* for *B. amyloliquefaciens* LBM 5006; F, *B. subtilis* ATCC 19659.

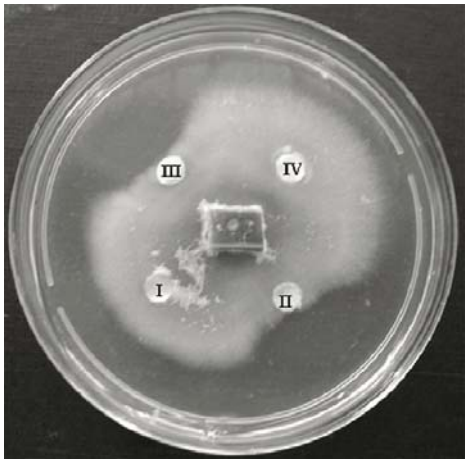

Fig. 2. Inhibitory activity of *B. amyloliquefaciens* LBM 5006 against *F. oxysporum f. licopersici*. Aliquots (10 µl, 3200 AU/ml) of the test samples were added to the peripheral disks and plates were incubated at 30°C for 48 h. (I) Crude supernatant, (II) partially purified, (III) purified, and (IV) negative control (phosphate buffered saline).

The antimicrobial activity was stable in the pH range from 2 to 11, was resistant to heating at temperatures higher than 80°C for 30 min, and by treatment with all the proteolytic enzymes tested. The substance was reactive with ninhydrin.

The UV absorption spectrum of the purified substance was examined between 190 and 340 nm. The purified peptide showed absorbance maxima at 198 and 210 nm, and there is no appreciable absorbance above 300 nm (Fig. 3A). Minor absorbance peak was also observed at 270 nm. Intense signal was observed at lower than 210 nm, typical of protein absorption. A shoulder at 220 nm is corresponding to characteristic absorption of peptide bonds.

The infrared spectrum is shown in Fig. 3B. Characteristic absorption bands at 1553, 1653, and 3250 cm^{-1} indicate that the substance contains peptide bonds. Bands that result from C-H stretching (2950, 2850, 1454, 1428 cm^{-1}) indicate the presence of aliphatic chain(s). A lactone ring is suggested by the absorption at 1740 cm^{-1}.

In order to determine accurately the molecular mass of the antimicrobial peptide, mass spectroscopy analysis was carried. The mass spectrum revealed two clusters with major peaks at *m/z* 1058 Da and 1464 (Fig. 4). The cluster of 6 peaks that were observed at *m/z* 1506, 1492, 1477, 1464, 1450, and 1436, differed by 14 Da each other. The other cluster showed peaks at *m/z* 1090, 1074, 1058, 1044, 1036, and 1022.

Discussion

The soil isolate *B. amyloliquefaciens* LBM 5006 produces an antimicrobial substance which inhibits several pathogenic and food-spoilage bacteria, such as *L. monocytogenes*, *B. cereus*, *Serratia marcescens*, and *Pasteurella haemolytica* (Lisboa *et al*., 2006). In this study the antimicrobial activity of strain LBM 5006 was observed against most phytopathogenic fungi tested, implying it has great potential for biological control of many fungal diseases. The potential of some strains of *Bacillus* spp. to synthesize a wide variety of metabolites with antifungal and/or antibacterial activity has been described (Souto *et al*., 2004; Chen *et al*., 2008).

Antimicrobial substance(s) from *B. amyloliquefaciens* LBM 5006 was isolated by gel filtration chromatography and 1-butanol extraction. The substance eluted near the void volume of Sephadex G-100 column, contrasting with the lower MW observed by mass spectroscopy. Discrepancy in MW determination has been shown for some bacteriocins and has been associated to the strong hydrophobic nature of the peptides (Oscáriz and Pisabarro, 2000). This behavior is similar to linocin M18 (Valdés-Stauber and Scherer, 1994) and iturin A_2 (Yu *et al*., 2002).

The ultraviolet and FITR spectra of the purified antimicrobial substance offer valuable information about its structure. The ultraviolet spectrum is compatible with a polypeptide. In agreement, most peptide antibiotics produce the characteristic

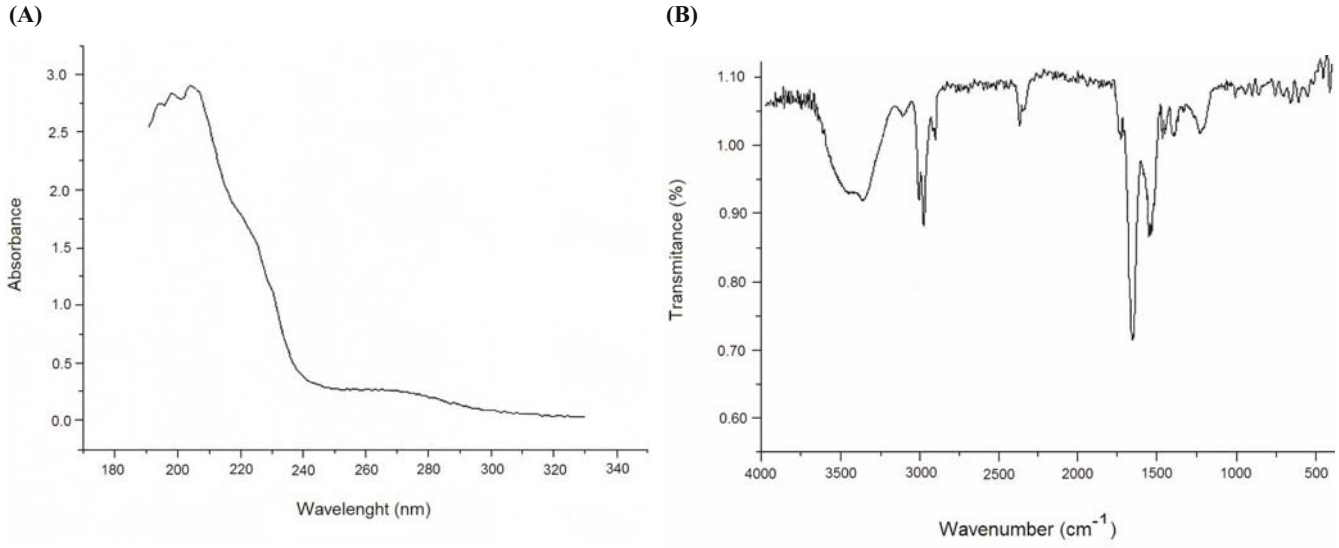

Fig. 3. Ultraviolet (A) and FTIR (B) spectra of purified antimicrobial substance produced by *B. amyloliquefaciens* LBM5006.

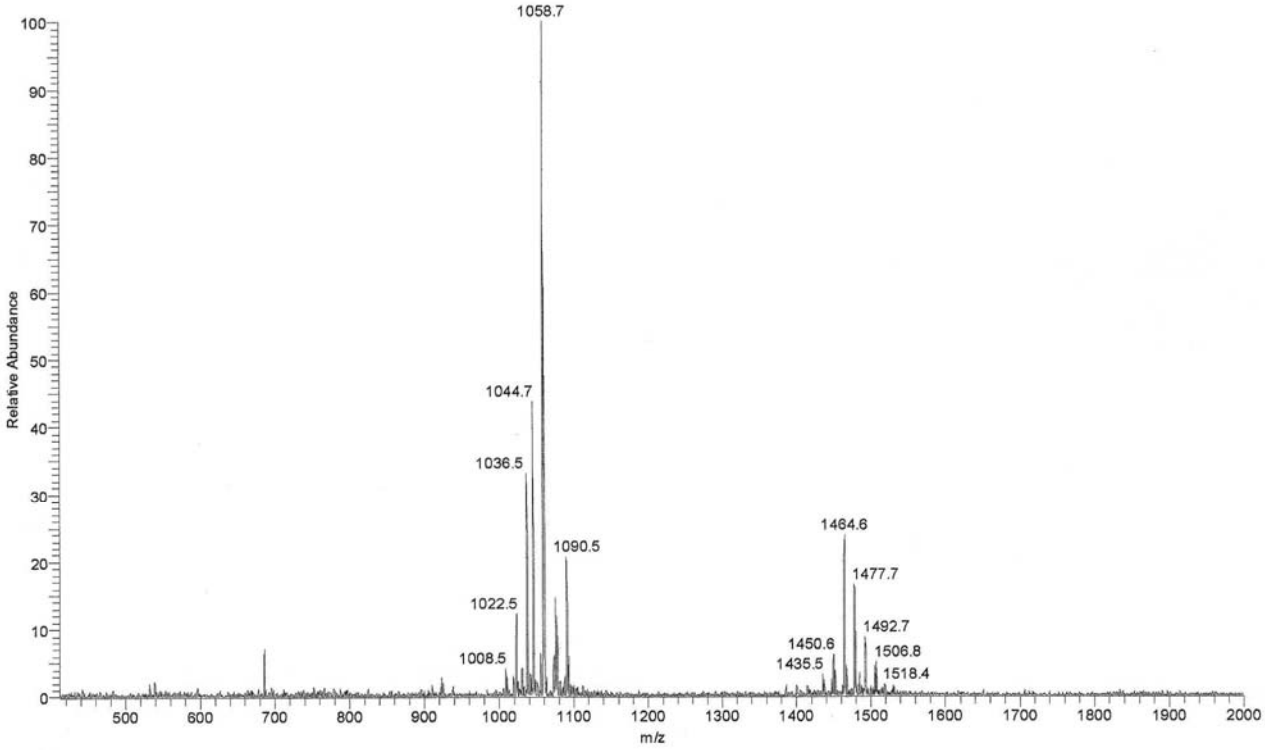

Fig. 4. MALDI-TOF spectrum of purified antimicrobial substance produced by *B. amyloliquefaciens* LBM5006.

absorbance pattern of absorbance maxima at 210-230 and 270-280 nm (Kurusu and Ohba, 1987).

Analysis of the FTIR spectrum shown typical absorption bands corresponding to N-H stretching of proteins and peptide bonds (Maquelin *et al*., 2002), concrete evidence that the substance contained a peptide in its structure. The presence of an absorption band at 1653 cm^{-1} associated with absorption between 3500 and 3200 cm^{-1} indicate amide functional group. The band at 1653 cm^{-1} associated also with the band at 2925 cm^{-1} indicate the carboxyl group (Maquelin *et al*., 2002). The presence of carboxyl and amino groups indicate the existence of amino acids, suggesting a polypeptide structure. Two prominent spectral bands in the region between 1800 and 1500 cm^{-1} corresponded to amide I (1653 cm^{-1}) and amide II (1553 cm^{-1}) (Fabian *et al*., 1993). The first band arose from the vibrational stretching of the chain of C=O group and the second doubt to the vibration of the bond N-H strongly linked to the stretching of C-N bond. The absorption in the region between 1600 and 1700 cm^{-1} appears, mainly, doubt to the vibrational stretching of carbonyl group of peptide bonds (Gaussier *et al*., 2003). Additional absorption valleys indicating aliphatic chains may be related with predominance of hydrophobic amino acids such as Val, Leu, Ile, or it contains a fatty acid in their structure. Antimicrobial activity of lipopeptides produced by *Bacillus* spp. has been described (Yakimov *et al*., 1995; Souto *et al*., 2004). Such peptides belong to a subclass of peptolides, cyclic peptides that contain fatty acids, often presenting less than 10 amino acids and molecular mass as low as 1500 Da (Vater *et al*., 2002). In addition, there is evidence that a lactone ring is present in this case, typical of fengycin families of lipopeptides.

MALDI-TOF has been used as an efficient tool for identifycation of antimicrobial peptides, including hydrophobic lipopeptides in the range of 1-1.5 kDa (Stein, 2008). The analysis of the mass spectrum of antimicrobial peptides of *B. amyloliquefaciens* LBM 5006 indicated that the peak at *m/z* 1464 correspond to C16-fengycin A (Hu *et al*., 2007). These peaks differ by 14 Da, suggesting a series of homologous molecules or fragments having different length of fatty acid chain (CH_2=14 Da). The peak at *m/z* 1506 matches with C17-fengycin B, which has the amino acid Val instead Ala at position 6 (Hu *et al*., 2007). The main peak at *m/z* 1058 Da agrees with iturin E (Besson and Michel, 1987) and isomers of iturin A described for *B. amyloliquefaciens* RC-2 (Hiradate *et al*., 2002) and *B. amyloliquefaciens* CCMI 1051 (Caldeira *et al*., 2008), and the surround peaks were deduced to be isoforms with different fatty acid side chains. This *m/z* is also close to C15-bacillomycin L $[M+Na]^+$ and C15-surfactin $[M+Na]^+$ (Stein, 2008), although the strain LBM 5006 lacks the functional *sfp* gene to produce surfactin. Positive reaction with ninhidrin also indicates free amino groups in the constituent amino acids, like Asn and Gln that are present in iturin A and fengycin, respectively. These results suggest that antifungal activity of strain LBM 5006 is associated with the lipopeptides iturin and fengycin. The co-production of different lipopeptides by *Bacillus* spp. may difficult the purification. In addition, the structural variability makes it complicated to definitively identify different families of lipopeptides and their homologues (Akpa *et al*., 2001; Caldeira *et al*., 2008; Chen *et al*. 2008).

The presence of gene *ituD*, related to production of iturin, and absence of the surfactin-related gene *sfp*, was observed in *B. amyloliquefaciens* LBM 5006. Both peptides are synthesized

by the action of large multienzyme complex, and these genes are essential to their respective production (Schneider *et al*., 1998; Stein, 2005). *B. amyloliquefaciens* is known to produce iturins, a family of cyclic lipopeptide antibiotics (Hiridate *et al*., 2002). *B. amyloliquefaciens* and *B. subtilis* are closely related and a diversity of lipopeptide antibiotics, such as surfactins, iturins, bacillomycins and mycosubtilin, are produced by *B. subtilis* (Stein, 2005), whose production is thought to be under complex regulation (Duitman *et al*., 1999; Yao *et al*., 2003). Our results indicate that *B. amyloliquefaciens* LBM5006 produces a mixture of antimicrobial peptides during cultivation in BHI broth as well.

Iturin A production is often an important factor conferring antifungal activity to the *Bacillus* strains. Iturin and fengycin lipopeptides have been assigned as key factors in antagonism of *B. subtilis* toward *Podosphaera fusca*, an ectoparasite causing powdery mildew in many cucurbits (Romero *et al*., 2007). In our study, *B. amyloliquefaciens* LBM 5006 showed inhibitory activity against *Aspergillus phoenicis*, *Bipolaris sorokiniana*, *F. oxysporum f. licopersici*, suggesting that the broad inhibitory activity observed may be associated to the action of iturin-like and fengycin-like peptides.

The development of fungicide-resistant pathogens, the detection of undesirable chemical residues in the food chain and ban of some of the most effective fungicides have intensified the search for safer approaches to efficiently control pathogenic fungi infections (Winteringham, 2008). Among the alternatives, biological control through the use of natural antagonistic microorganisms has been extensively studied and some *Bacillus* strains have been shown to be effective against various pathogens (Yu *et al*., 2002). Antimicrobial peptides produced by *Bacillus* spp. have been proposed as biocontrol agents against several phytopathogenic microorganisms (Bais *et al*., 2004; Cladera-Olivera *et al*., 2006). To sum up, this study identified an isolate showing significant activity against phytopathogenic fungi, showing potential use for the production of antifungal preparations.

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