PAPER

PRELIMINARY CHARACTERIZATION OF A BACTERIOCIN-LIKE SUBSTANCE PRODUCED BY A *BACILLUS SUBTILIS* ISOLATED FROM ARGENTINEAN VEGETABLE FOOD

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ABSTRACT

Bacillus spp. produces a large number of antimicrobial peptides and includes a variety of species of industrial importance. The aim of this study was to characterize the antimicrobial activity associated with a *Bacillus subtilis* strain. The activity of the crude bacteriocin-like substance (CBLS) was dependent on the components of the culture media and was detected in the exponential phase of growth. The CBLS was stable at a variable pH range and at 100°C, but it was inactivated by sterilization conditions. It was also stable to storage at refrigeration and freezing temperatures for a considerable time. The mode of action of the antimicrobial activity was bacteriostatic and the effect on the cell membrane was determinated by Fourier transform infrared (FTIR) spectroscopy.

- Keywords: antimicrobial peptides, *Bacillus cereus*, *Bacillus subtilis*, bacteriocins -

INTRODUCTION

Due the concern on reducing the use of chemical additives in food, since some of them have been proven harmful to human health as well as the environment, the necessity of more natural and safe food products has been recognized (XU *et al.*, 2013). Bacteriocins are an alternative for natural food preservation, as well as for human therapy as possible additive or replacements for currently used antibiotics (ABO-AMER, 2007).

Bacteriocins are proteinaceous compounds bioactive to other bacteria than the producing strain (JOERGER, 2003). The activity spectrum of bacteriocins can be narrow and limited to closely related species, or it can be relatively extensive and comprise many different bacterial species (OSCARIZ and PISABARRO, 2000).

The isolation of new strains also allows to discover new bacteriocin produced by them, since different kinds of bacteria produce different types of bacteriocins. Consequently due to the nascent field of biopreservation there is a demanding need to explore and isolate more and more bacteria from new sources capable of producing novel bacteriocins and characterize them to be added to food (SHARMA and GAUTAM, 2008).

Bacillus spp. is of major interest in bacteriocin research since this genus produces a diverse array of antimicrobial peptides with several different basic chemical structures (CLADERA-OLIVERA *et al.*, 2004; XIE *et al.*, 2009; ABRIOUEL *et al.*, 2010). The *B. subtilis* group is one of the major producers of these substances (MARTIRANI *et al.*, 2002; CLADERA-OLIVERA *et al.*, 2004; LISBOA *et al.*, 2006; ANTHONY *et al.*, 2009; HAMMAMI *et al.*, 2009; XIE *et al.*, 2009; KINDOLI *et al.*, 2012), being *B. subtilis* one of the most prolific. In addition, *B. subtilis* is generally recognized as safe organisms (GRAS) by the Federal Drug Administration (FDA) for specific applications such as enzyme production (APETROAIE-CONSTANTIN *et al.*, 2009).

The extensive screening of nearly 100 strains of *Bacillus* spp. isolated from vegetable food from Argentina led to the selection of several strains which demonstrated antibacterial activity (FANGIO *et al.*, 2010).

This paper reports a preliminary characterization of the biological and physicochemical properties of the antimicrobial substances produced by a strain isolated from rice, *B. subtilis* R1.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacillus subtilis R1 isolation and bacteriocin-like identification have been described elsewhere (FANGIO *et al.*, 2010). Indicator and producer strains were kept frozen in 20% (v/v) glycerol at –20°C until use. *Bacillus* spp. were growth in nutritive agar and *Paenibacillus larvae* was cultured in Mueller-Hinton, yeast extract, glucose, sodium pyruvate and agar (MYPGP) supplemented with nalidixic acid at 37°C for 48 h.

Preparation of crude extracts

The bacteriocin-like producing strain was grown in brain-heart infusion (BHI) at 37°C for 24 hours. After incubation, the bacterial cells were removed by centrifugation at 10,000 g for 15 min. The supernatant was collected and filtered with membrane 0.22 µm (Titan syringe filters; Sri Scientific Resources Inc., USA). This supernatant was designated as crude bacteriocin-like substance (CBLS) (XIE *et al.*, 2009).

Reagents and media

Mueller-Hinton Agar (MH), nutrient broth (NB) and brain-heart infusion (BHI) were from Britania (Buenos Aires, Argentina). All other media and reagents were from Merck (Darmstadt, Germany). Mueller-Hinton, yeast extract, glucose and piruvato of sodium agar (MYPGP) were prepared according to composition reported by DINGMANN and STAHLY (1983).

Bacteriocin-like activity assay

Antimicrobial activity of the supernatant was evaluated by the agar well-diffusion method (FANGIO *et al.*, 2010). *P. larvae* was chosen as indicator strain because of its high sensitivity to the antimicrobial substance (FANGIO *et al.*, 2010). In addition, a *B. cereus* strain was selected as indicator strain since it is an important foodborne pathogen. The CBLS titre was determined by the serial two-fold dilution method described in LISBOA *et al.* (2006) and expressed as activity units (AU) per milliliter.

Kinetics of growth strains and antimicrobial activity production

To study the kinetics of production of the antimicrobial substance, the producing strain was seeded in 5 mL of brain heart broth and incubated 18 hours at 35°C. Fifty ml of BHI were inoculated with 1 mL (2 %) of the preinoculated broth and incubated at 35°C. Samples were taken at different times. To monitor the cell growth viable cells count was performed on nutrient agar plate, while production of antimicrobial substance of the CBLS was measured by the agar well-diffusion method (CHERIF *et al.*, 2001).

Effect of culture media

Different commercial culture media (brain heart infusion (BHI), lactose broth, Mueller-Hinton broth and nutrient broth) were analyzed as substrates for the production of antagonis-

tic substances. The strain was seeded in 5 mL of BHI and incubated 18 hours at 35°C. An aliquot of this preinoculated culture was added in every culture media and incubated at 35°C for 18 hours. The antimicrobial activity of the CBLS obtained from every culture media was analyzed using the agar diffusion assay (DOMINGUEZ *et al.*, 2007).

Effects of pH and heat on antimicrobial activity

To determine the stability of the antimicrobial substances to pH, CBLS was diluted in different buffers and incubated at room temperature for 2 hours. To analyze the heat stability of the substances, CBLS were subjected to the following treatments: 20°, 40°, 60° and 80°C for 30 min, 100°C for 5, 10, 15, 20, 30, 40, 50 and 60 min and 121°C at 1 atm for 15 minutes. After treatments, the antimicrobial activities were evaluated using the agar diffusion method (LISBOA *et al.*, 2006).

Stability in different storage conditions

CBLS was stored under refrigeration (4°C) and freezing (-20°C). At different time intervals (10 and 30 days) the stored material was evaluated for antimicrobial activity by agar diffusion method (ABO-AMER, 2007).

Kinetics of antimicrobial action

To study the mode of action of the antimicrobial substance, CBLS (75 AU/mL) was added to fresh cultures of *B. cereus* in brain heart broth in the logarithmic phase of growth. Samples were taken at different times and recorded both cell biomass (measured as OD_{600nm} with a spectrophotometer UV-Vis Spectrophotometer Scanning Shimadzu (UV-2101PC)) and viable cells count on nutrient agar plate (XIE *et al.*, 2009).

Phospholipase C activity and capacity for erythrocyte hemolysis

Phospholipase C activity and capacity for erythrocyte hemolysis of CBLS were verified according to BIZANI *et al.* (2005).

Fourier transform infrared (FTIR) spectroscopy

A bacterial suspension of the indicator bacteria *Paenibacillus larvae* (10^8 cfu/mL) was added to an equal volume of CBLS. After incubation for 1.5 hours, both treated and control cells were washed three times with sterile distilled water. Twenty µL of each bacterial sample was applied onto a ZnSe optical plate, dried and then analyzed by FTIR spectroscopy. All IR spectra (4,000-400 cm^{-1}) were obtained according to BIZANI *et al.*, 2005. To minimize the variation due to the difference between different biomass samples, the spectra were normalized according to DEAN *et al.* (2010).

Concentration of inhibitory substance

CBLS was concentrated by extraction with organics solvent using 1-butanol (BIZANI *et al.*, 2005). After evaporation of the 1-butanol, the extract was suspended in phosphate buffer pH 7.2 and stored at 4°C until use. The measure of the activity of the extract was determined by the agar diffusion method.

Statistical analysis

The antimicrobial activity of strains in the different treatments were compared using analysis of variance (ANOVA) with Tukey's test (ZAR, 2009) using SPSS 15.0 (SPSS Inc., Chicago, Illinois, USA) for Windows.

RESULTS AND DISCUSSION

In a previous paper (FANGIO *et al.*, 2010) we reported the identification of a new bacteriocin-like substance produced by *B. subtilis* R1. In this report, the proteinaceous nature of this substance produced by *B. subtilis* R1 was established. However, its biochemical characterization was not completed.

The antibacterial activity was detected in the mid-exponential phase with a maximum in the stationary phase (Fig. 1), which suggests that the antimicrobial peptide is produced as a primary metabolite (CLADERA-OLIVERA *et al.*, 2004). The kinetics of this antimicrobial peptide was similar to the bacteriocin produced by *B. subti-*

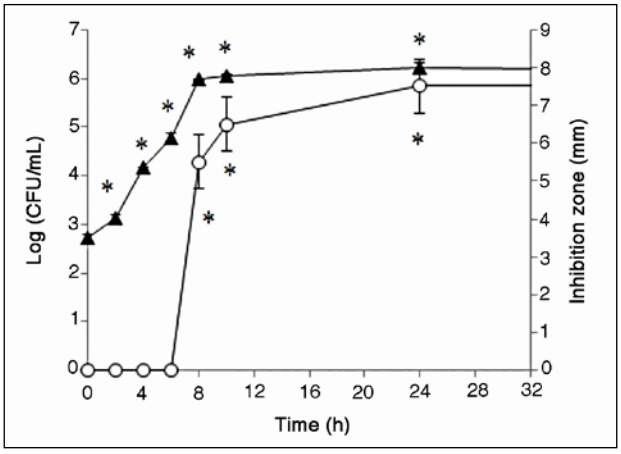

Fig. 1 - Kinetics of antimicrobial substance production during the growth of *B. subtilis* R1 in BHI at 37°C for 32 h. Viable cell count (log CFU/mL) of *B. subtilis* R1 (black triangles) and antimicrobial activity expressed as inhibition zones (mm) against *B. cereus* (open circles). An asterisk indicates statistically significant difference ($p < 0.05$) with initial values.

lis LFB112 (XIE *et al.*, 2009) which antibacterial activity was detected in half of the rapid growth phase and peaked in early stationary phase. The comparative study of the kinetics of growth of the strain and production of the antimicrobial substance is important to identify the most effective production stage.

Nutrient selection and determination of their concentrations in the culture media is an important step in cell growth and production of useful metabolites produced from microorganisms. To optimize the conditions of the culture medium is especially important for products that are required in large amounts as for technological uses (DOMINGUEZ *et al.*, 2007). The inhibitory activity of CBLS obtained with different culture media showed that CBLS obtained from the growth of *B. subtilis* R1 in brain heart broth, produced a higher activity ($p<0.05$) that those obtained from the other culture media studied (Fig. 2). Brain heart broth is a rich culture medium that differs from other media analyzed for possessing large quantities of beef brain heart infusion. An antimicrobial substance produced by a strain of *B. amyloliquefaciens* presented important activity when is grown in brain heart broth, but not in peptone broth, suggesting that the biosynthesis of the peptide requires a rich medium (LISBOA *et al.*, 2006). By contrast, peptone seems to be a key nutrient for the production of antifungal compounds by *B. amyloliquefaciens* RC-2 (YOSHIDA *et al.*, 2001). Mueller-Hinton broth, also has large quantities of beef extract and caseine hydrolysate, but instead of glucose, the main source of carbohydrate is starch. In studies of production of bacteriocins of lactic acid bacteria has been assumed that the supply of amino acids promotes the production of bacteriocins (AASEN *et al.*, 2000) and also the lack of glucose has been considered a limiting factor for the production of bacteriocins (RUSSELL and MANTOVANI, 2002). In addition, JATINDER and JANA (2009) observed that a concentration increased from 10 to 15 g/L of starch and from 2 to 5 g/L of peptone has negative effects on cell concentration of a *Bacillus licheniformis* strain.

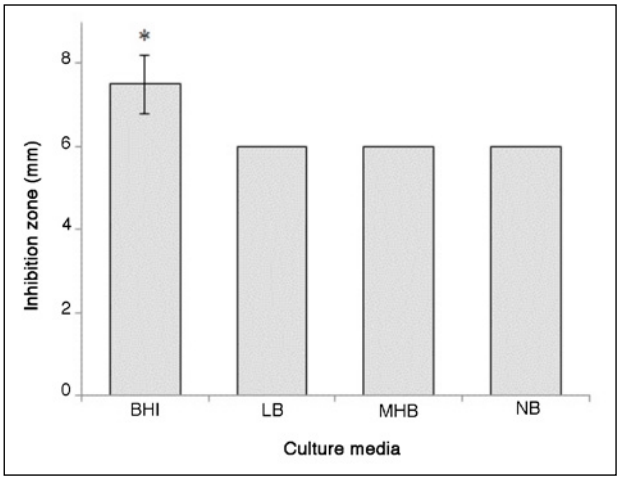

Fig. 2 - Effect of culture media on the production of antagonistic substances: brain heart infusion (BHI), lactose broth (LB), Mueller-Hinton broth (MHB) and nutrient broth (NB). The antimicrobial activity of different extracts was measured by the agar diffusion method against *B. cereus* and expressed as inhibition zones (mm). An asterisk indicates statistically significant difference ($p< 0.05$).

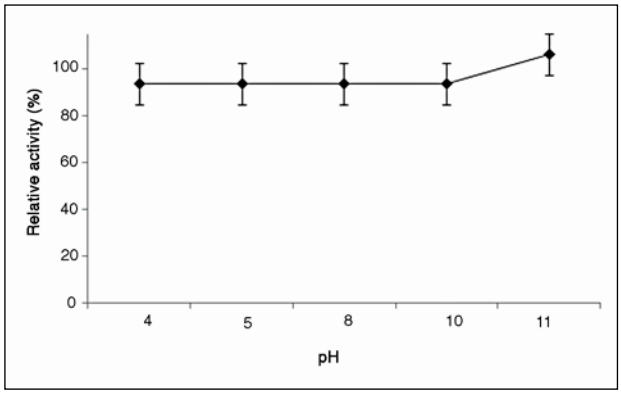

Fig. 3 - Effect of pH on the antimicrobial activity of CBLS. The antimicrobial activity is expressed as the percentage of relative activity against *B. cereus* of the buffered extracts compared with the untreated extracts of pH 7. An asterisk indicates statistically significant difference ($p< 0.05$).

CBLS produced by strains of *B. subtilis* R1 was also active over a wide pH range (Fig. 3), showing not significant differences ($p<0.05$). It was observed that this CBLS was stable at pH 4 to 11, without showing reductions of activity in the extreme pH. The bacteriocin Bac 14B produced by a *B. subtilis* strain was studied in the pH range 2 to 12, showing an optimal activity at pH 7 and a significantly reduction of the activity to less than 2.5 times that of the control at pH 9 (HAMMAMI *et al.*, 2009). In some cases, the effect of pH has been associated with changes in the activity of enzymes involved in post-translational modification or pH-dependent release of bacteriocins from the cell surface (RUSSELL and MANTOVANI, 2002). The stability of bacteriocins at different pHs is a great advantage from the industrial point of view, since the pH of many foods varies from acidic to very basic (MARTIRANI *et al.*, 2002). These observations increase the probability that these compounds are suitable for the food conservation and personal care applications, because they can adapt and function in a variety of environments (SUTYAK *et al.*, 2008). The importance of pH on bacteriocin activity has been explained due to its impact on the stability and solubility of the bacteriocins (LIU and HANSEN, 1990). For example, nisin has shown that it is 228 times more soluble at pH 2 than at pH 8 (LISBOA *et al.*, 2006) and has a much higher activity at pH values below 6.

The antimicrobial substance produce by *B. subtilis* R1 was partially stable to heat treatments; the activity was maintained during treatments up to 100°C and disappeared only after

15 min of autoclaving at 121°C (Figs. 4 and 5). Studies of thermal effect on the antimicrobial activity revealed that CBLS activity resisted high temperatures. At 100°C the CBLS remained active despite it does not resist sterilization conditions. Similar results were obtained for bacteriocin substance of *B. subtilis* 14 whose activity resisted up to 2 hours at 100°C (HAMMAMI *et al.*, 2009) or the bacteriocin of *B. amyloliquefaciens* described by SUTYAK et al. (2008) that resists heat treatment up to 100°C for 60 min. However, a CBLS produced by *B. subtilis* strain loses most of its activity at 100°C for 10 min (KINDOLI *et al.*, 2012). The effect of temperature on the antimicrobial activity of these substances is important for their potential use as food additives. The technological processes applied to foods can affect the inhibitory activity of these compounds. Food tolerates a variety of heat treatments during preparation, so it is imperative that antimicrobial peptides were resistant to heat when food production demands this kind of treatment.

The effect of both pH and temperature on the antimicrobial substance showed that CBLS maintain the antimicrobial activity at high temperatures and mainly acidic pHs (Fig. 6).

The crude extracts produced by *B. subtilis* R1 exhibited antimicrobial activity under conditions of freezing and cooling after 10 and 30 days of storage in the refrigerator. CBLS maintained an activity above 80% compared to the initial activity (Fig. 7). The antimicrobial activity of CBLS was stable to storage at refrigeration and freezing temperatures for a considerable time. ABO-AMER (2007) obtained similar results in storage studies of plantaricin AA135 produced by *L. plantarum* AA135 determining that this bacteriocin crude extract stored at -20° and 4°C conserved its activity without substantial loss for a minimum of 100 days. The fact that this substance show stability after freezing has a technological importance, since the demand of frozen food is increasing, which is associated with problems as maintaining freezing temperature during storage and distribution. These results suggest that this antimicrobial substance could be applied to foods that have a prolonged durability at refrigerating or freezing temperatures, without showing a reduction of its antimicrobial action.

The mode of action of bacteriocins may be bactericidal or bacteriostatic, determining death or extension of lag phase, respectively (SETTANI and CORSETTI, 2008). Indicator bacteria cells in exponential growth are subjected to different concentrations of bacteriocins, assessing the viability and cell biomass. A reduction in viable cell counts and OD_{600nm} of *B. cereus* treated with the CBLS was showed (Fig. 8). After 2 hours of treatment the viable cell counts showed a significant reduction, compared to the control at the same time of incubation. Also it was measured as a reduction of OD_{600nm} of the treated cells compared with the control at 6 h of treatement. CBLS produced by *B. subtilis* R1 showed inhibitory activity against closely related species (FANGIO *et al.*,

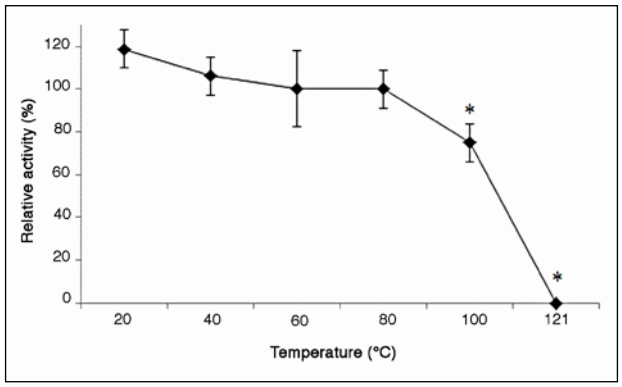

Fig. 4 - Effect of temperature on antimicrobial activity. The antimicrobial activity is expressed as the percentage of relative activity against *B. cereus* of the extracts incubated 30 minutes at different temperatures and 15 minutes at 121°C compared with untreated extracts incubated at 37°C. An asterisk indicates statistically significant difference ($p < 0.05$).

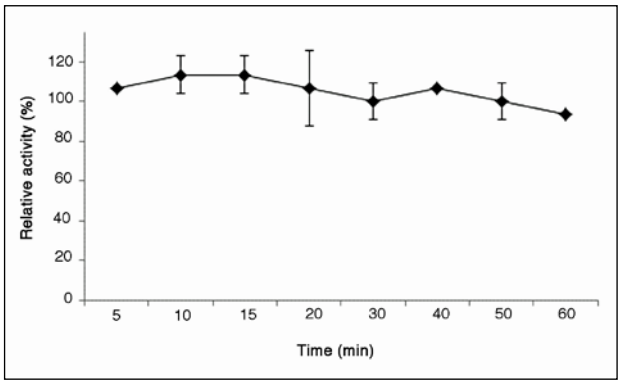

Fig. 5 - Effect of exposure time at 100°C on the antimicrobial activity. The antimicrobial activity is expressed as the percentage of residual activity against *B. cereus* of extracts incubated at different times at 100°C compared to the antimicrobial activity of extracts. An asterisk indicates statistically significant difference ($p < 0.05$).

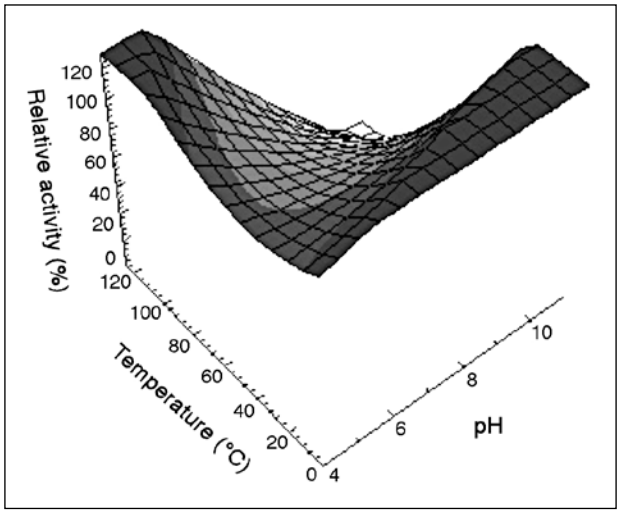

Fig. 6 - Effect of pH and temperature on the stability of *B. subtilis* R1 bacteriocin-like substance.

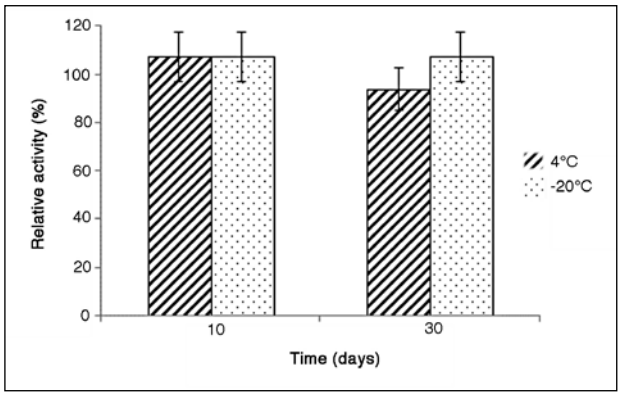

Fig. 7 - CBLS stability in different temperatures and storage times. The antimicrobial activity is expressed as the percentage of relative activity of the extracts stored compared to untreated extracts against *B. cereus*. An asterisk indicates statistically significant difference ($p < 0.05$).

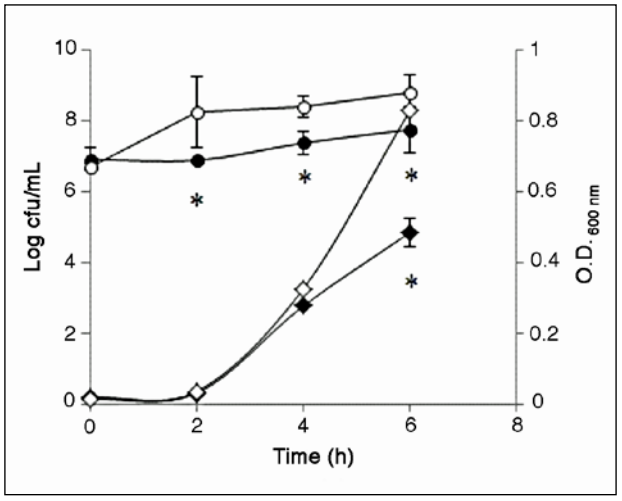

Fig. 8 - Effect of CBLS on the growth of *B. cereus*. Optical density (diamonds) and log CFU/mL (circle) of *B. cereus* untreated (open) and treated (closed) with CBLS. An asterisk indicates statistically significant difference ($p < 0.05$) between control and treated cells.

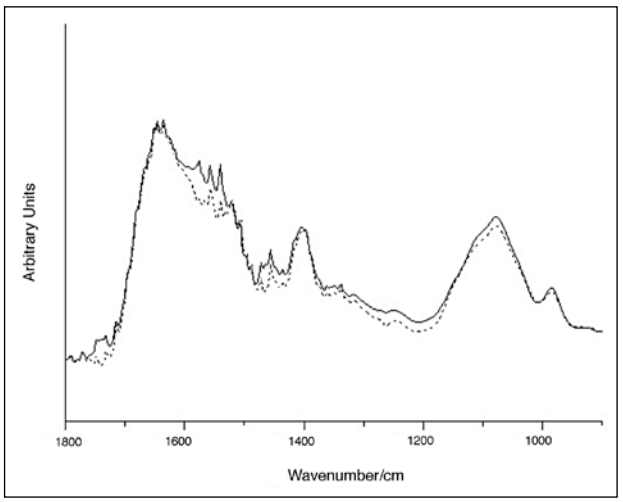

Fig. 9 - Spectroscopy Fourier Transform Infrared (FTIR) of *P. larvae*. The normalized infrared spectra (1,800-900 cm^{-1}) of treated (dashed line) or untreated (solid line) biomass were recorded using the ATR method.

2010). In this study viable cell counts of the indicator strain *B. cereus*, were immediately affected by the presence of the supernatant tested, showing a reduction in the rate of growth of *B. cereus* compared to the untreated control. Consequently our results suggest that this substance has a bacteriostatic effect against *B. cereus*. However, the effect could be subject to the particular assay conditions, such as the quantity and purity of the antimicrobial substance, the indicator strain, and its cellular concentration. Indeed, some bacteriocin has showed a bacteriostatic activity in a low dose while a bactericidal and bacteriolytic activity was observed at high concentration of the bacteriocin (BOUCABEILLE *et al.*, 1997).

The hemolytic activity of CBLS R1 assayed against erythrocytes was negative. In addition, negative reactions for phospholipase C were also observed. Gram-positive bacteria produce a variety of extracellular molecules that show antibacterial activity, including hemolysins, phospholipases and muramidases (OSCARIZ and PISABARRO, 2000). Some bacteriocins produced by bacteria isolated from food, have hemolytic activity but this activity is not associated with antimicrobial activity (BIZANI *et al.*, 2005). On the other hand class III bacteriocins include megacins A-216 and A-19 213 large proteins (430 kDa) with phospholipase activity as produced by strains of *B. megaterium*. BIZANI *et al.* (2005) determined the lack of phospholipase activity associated with the proteolitic enzyme inactivation suggesting the protein status of the cerein 8A.

The effect of the antimicrobial substance over macromolecular structures of the cell was studied by FTIR spectroscopy using *P. larvae* as testing strain. The cells of *P. larvae* treated with the CBLS and the control showed differences in the absorbance in the band corresponding to the region of fatty acids (3,000 to 2,800 cm^{-1}) (data not showed), in the band assigned among other to ester carbonyl and carboxyl groups (1,800-1,500 cm^{-1}), and in the band assigned among others to the phospholipids (1,500-1,200 cm^{-1}) (NIETO *et al.*, 2004). FTIR was used to obtain a better understanding of the molecular mechanisms responsible for the antimicrobial activity of the CBLS. FTIR spectroscopy applied to cultures of *P. larvae* treated with the CBLS shows significant changes in symmetrical bonds of C=O (1,400 cm^{-1}), antisymmetrical stretching of bonds CH_2 and CH (2,918 cm^{-1}) and antisymmetrical stretching of bonds of CH and CH_3 (2,955 cm^{-1}) in the region corresponding to fatty acids, essential components of the membranes. Some authors proposed that the effects on cell membranes eventually translate into the loss of vesicular contents across the local destabilization of lipid packing, or the formation of "pores". This effect would modify the barrier properties of the membranes due to damage in areas where the antimicrobial substance interacts with the phospholipids. This would cause

structural fluctuations that may well explain the main mode of action that quickly affects membrane integrity instead of other vital processes. Similar results were obtained by BIZANI *et al.* (2005) when they studied the mechanism of action of cerein 8A by FTIR, proposed that it could be a peptide that forms pores.

The CBLS was partially purified by extraction with solvents, in this case, 1-butanol, resulting in a concentrated solution. The concentrated extract showed higher activity that the crude extract against *B. cereus* and *P. larvae* (Table 1). The titration of the antimicrobial activity of the concentrated extract against *B. cereus* was measured as 400 AU/mL. Many methods have

Table 1 - Antimicrobial activity of extracts.

	B. cereus	P. larvae
CBLS	8,3±0,6[a]	15,7±0,6[a]
Concentrated extract	14,5±0,7[b]	34±1,41[b]

*Antimicrobial activity of different extracts is expressed as inhibition zones (mm). Values against the same indicator strains (vertical columns) with different superscripts differ significantly ($P < 0.05$).

been used to concentrate and purify the bacteriocins, some of which combine several procedures (LI *et al.*, 2006), while others use just a few steps of purification (OSCARIZ and PISABARRO, 2000; ABO-AMER, 2007). When these methods were performed, the recovery of antimicrobial activity should take into account, since in each step of concentration and purification material loss occurs. As a result, we obtain a concentrated extract which antimicrobial activity is greater than the original crude extract. TEN BRINK *et al.* (1994) determined that the bacteriocin acidocin B is a hydrophobic peptide due to recovery with 1-butanol extraction from cell-free neutralized supernatants, being a feature shared with other bacteriocins (KLAENHAMMER, 1993).

CONCLUSIONS

The activity of the crude bacteriocin-like substance (CBLS) was dependent of the components of the culture media and was detected in the exponential phase of growth. The CBLS was stable at a variable pH range and temperature of pasteurization, being inactivated by sterilization. In addition, CBLS was stable to storage at temperature of refrigeration and freezing. The mode of action of the antimicrobial activity was bacteriostatic and changes of the bands in the infrared region also make evident that the effect occurs on the cell membrane by FTIR. These results indicate that the BLS produced by *B. subtilis* R1 would have a technological interest in food preservation.

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