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Characterization and purification of bacteriocin produced by *Enterococcus* sp. GHB26 isolated from Algerian paste of dates "Ghars"

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Strain *Enterococcus* sp. GHB26, isolated from the Algerian paste of dates "Ghars", produced a bacteriocin. This bacteriocin was inactivated by proteolytic enzymes. Antibacterial activity of the bacteriocin was heat stable at 120°C for 20 min (533 AU/ml), stable at pH range of 2 to 12 and resistant to chemicals (SDS, EDTA, NaCl, Tween 80, Urea). The active bacteriocin from the cell-free supernatant of *Enterococcus* sp. GHB26 was purified by precipitation with ammonium sulfate followed by various combinations of gel filtration on a Sephadex G-25 column, cation exchange chromatography on a CM-Sephadex Cellulose column and reverse phase-high performance liquid chromatography on a C18 column. The bacteriocin was eluted as a single peak on the chromatogram from reverse phase-high performance liquid chromatography attesting the purity of this bacteriocin. The bacteriocin exhibited a bactericidal mode of action. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis indicated that molecular weight of this bacteriocin is close to 3.5 kDa.

Key words: Paste of dates, bacteriocin, antibacterial activity, chromatography, mode of action.

INTRODUCTION

Lactic acid bacteria had largely been exploited in the food fermentation and they display numerous antimicrobial activities (De Vuyst and Leroy, 2007; Šušković et al., 2010). Several lactic acid bacteria, mostly enterococci strains, are also found in the gastrointestinal tracts of humans and animals and are considered to exert health-promoting effects which include antimicrobial activity against pathogen strains. This is mainly due to the production of organic acids, diacetyl, hydrogen peroxide and acetaldehyde but also of other compounds, such as bacteriocins (Gürakan, 2007).

Bacteriocins are ribosomally synthesized peptides produced by bacteria that exhibit bactericidal or bacteriostatic effect against closely related bacteria but

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also against pathogenic bacteria (Collins et al., 2009). The bacteriocins are often cationic and amphiphilic or hydrophobic (Altuntas, 2013). The enterococcal bacteriocins are small heat stable proteins that kill closely related bacteria (Altuntas, 2013).

Bacteriocins from lactic acid bacteria have a wide potential applications such as, for example food biopreservatives and health care (Cotter et al., 2005; Šušković et al., 2010). Antimicrobial activity is often proposed as an important functional characteristic of probiotic strains, because bacteriocin biosynthesis by probiotics can contribute to eliminate undesirable bacteria during host colonization, thereby helping to prevent host from pathogen proliferation. Nisin and pediocin PA-1 produced by lactic acid bacteria are the only bacteriocins used in food preservation (Altuntas, 2013).

Before any use of a bacteriocin in food industry, its study must be done on biochemical and genetic characteristics, its antibacterial spectrum, its effectiveness in different food systems, and the regulatory implications (Javed, 2009). Each bacteriocin has unique properties and a particular efficiency in targeting microbial pathogens. This is why isolation and purification of new bacteriocins will be always beneficial (Elegado et al., 1997; Merzoug et al., 2015).The purification of bacteriocin uses different strategies depending on the properties of the producer bacteria and the experimental conditions available (Cintas, 1997), but it follows a common protocol that includes salt precipitation and various chromatography combinations like gel filtration, ion-exchange, hydrophobic-interaction, and reverse phase - high performance liquid chromatography from culture supernatants (Altuntas, 2013).

The aim of the present study was to characterize and investigate the antimicrobial activity of the cell-free supernatant of *Enterococcus* sp. GHB26 isolated from traditional date paste "Ghars" in different phases of bacteriocin purification. Furthermore, the interest was to find out the mode of action of the bacteriocin.

MATERIALS AND METHODS

Bacterial strains and media

In the present study, two strains from the Collection of the Laboratory of Biology of Microorganisms and Biotechnology, University of Oran, Algeria, were used: *Enterococcus* sp. GHB26 had been isolated from date paste "Ghars" (Dellali, 2012) and was used as producer of the bacteriocin and the strain *Enterococcus faecium* H3 (Lazreg et al., 2015), used as an indicator strain, had been isolated from "hammoum", a traditionally fermented barley. Strains were conserved by storage at -20°C in reconstituted skim milk (10% w/v). The strains were cultured in MRS broth medium (Fluka, Switzerland) at 30°C before use.

Detection of inhibitory activity

Bacteriocin screening was tested by the agar well diffusion method as described by Tagg and Mc Given (1971). 500 µL of a culture of the indicator strain *E. faecium* H3 (30°C, 18 h) was inoculated in 30 ml of MRS with 0.75% (w/v) agar and then poured onto plates. Aliquots of 60 µL from the cell-free supernatant, obtained after centrifuging a culture of the inhibitory strain *Enterococcus* sp. GHB26 (30°C, 18 h), were added to each well of the indicator lawn poured on MRS soft agar. After a diffusion step at 4°C overnight, the plates were incubated at 30°C for 24 h. The presence of the inhibition zone was considered as a positive result for bacteriocin production.

In order to exclude the inhibitory effect of lactic acid, the inhibitory strain *Enterococcus* sp. GHB26 was cultivated in MRS broth buffered with sodium phosphate buffer (0.1 M, pH 7). The activity of cell-free supernatant was defined as the reciprocal of the highest dilution value showing inhibition of the indicator strain and was expressed in arbitrary units per mililiter (AU/ml) (Graciela et al., 1995).

Physico-chemical characterization of the inhibitory substance

Preparation of cell-free supernatant

Enterococcus sp. GHB26 was cultivated 4% (v/v) in MRS broth buffered with sodium phosphate buffer (0.1 M, pH 7) and was grown for 18 h at 30°C. The cell-free supernatant was separated from cells by centrifugation at 3000 g for 15 min.

Effect of enzymes

Aliquots of 200 µL of the cell-free supernatant were incubated for 2 h at 37°C in the presence of 1 mg/ml of catalase, trypsin, pepsin, pronase E (Aktypis and Kalantzopoulos, 2003). All enzymatic solutions (Sigma-Aldrich) were prepared in buffer sodium phosphate (0.01 M, pH 7) except for pepsin which was dissolved in HCl of 0.02 M. Samples were boiled at 100°C for 5 min to stop the enzymatic reactions. Untreated cell-free supernatant was used as a control. The susceptibility of antibacterial activity to enzymes was evaluated by the agar well diffusion method (Tagg and McGiven, 1971).

Effect of temperatures

To determine the effect of temperature on the antibacterial activity, aliquots (200 µL) of cell-free supernatant were heated at 60, 80 and 100°C for 60 and 120 min and at 120°C for 20 min. The residual activity was evaluated according to Graciela et al. (1995) against *E. faecium* H3. The activity was expressed as arbitrary units (AU) per milliliter.

Effect of different pH

Aliquots of 200 µL of the cell-free supernatant were adjusted to different pH from 2 to 12 with HCl (5 M) or NaOH (5 M). After 4 h of incubation at 30°C (Hernandez et al., 2005), the residual antibacterial activity was tested as described before.

Effect of chemical agents

The chemical agents: SDS, Triton X-100, EDTA, NaCl, Tween 80 and Urea, were added separately (1% w/v) to the cell-free supernatant. After homogenization, the samples were incubated at 37°C for 5 h. Untreated cell-free supernatant was used as a control.

The residual antibacterial activity was tested by the agar well

diffusion method.

Purification of the bacteriocin GHB26

Precipitation by ammonium sulfate

Cell-free supernatant (140 ml) from *Enterococcus* sp. GHB26 was treated with ammonium sulfate to 40, 60 and 80% saturation. Mixtures were stirred at 4°C for 4 h and further centrifuged for 30 min at 3000 g. The precipitates were dissolved separately in 7 ml sodium phosphate buffer (0.02 M, pH 7) and investigated for their antimicrobial activity.

Sephadex G-25 Gel filtration chromatography

This chromatography separates molecules according to their size and it can be used for desalting. 1.2 ml of the precipitate obtained at 60% saturation of ammonium sulfate was applied to a Sephadex G-25 gel filtration column (26 cm length, 1.4 cm diameter, 5000 Da exclusion limit) that had been previously equilibrated with 0.1 M sodium phosphate buffer (pH 7). Elution was conducted using 0.1 M phosphate buffer at pH 7 at a flow rate of 0.5 mL/min. Fractions of 1 mL were collected and the absorbance measured at 280 nm. The inhibitory activity was determined by the agar well diffusion method. The active fractions were mixed and lyophilized.

Cation exchange chromatography on a CM- Sephadex Cellulose column

This column separates the peptides according to their electric charge at definite pH. The lyophilisate obtained from the gel filtration chromatography was suspended in 500 µL sodium phosphate buffer (0.1 M, pH 7) and separated by cation exchange chromatography through CM-Sephadex Cellulose column (10 cm length, 1.2 cm diameter) that had been previously equilibrated with 0.05 M sodium phosphate buffer (pH 7). Elution was conducted by a continuous gradient-elution (0-0.1 M NaCl, 0.1-1 M NaCl in 0.05 M phosphate buffer pH 7.0), at a flow rate of 0.5 ml/min. Fractions of 1 mL were collected and the absorbance measured at 280 nm. The inhibitory activity was determined and the active fractions were mixed and lyophilized.

Purification by reverse phase-HPLC

The lyophilisate obtained from the cation exchange chromatography was re-suspended in 250 µL of 0.1 M sodium phosphate buffer at pH 7 containing 10% (v/v) acetonitrile and purified by reverse phase-high performance liquid chromatography (RP-HPLC) on a column Inertsil ODS2 C18 (250 mm length, 4.6 mm internal diameter, 5 µm particle size). Elution was performed using a gradient of acetonitrile from 0 to 100% in 0.1% aqueous trifluoroacetic acid (TFA) for 30 min, at a flow rate of 1 mL/min. Eluted peaks were detected at 226 nm and the corresponding fractions were collected. The active fractions were mixed and lyophilized, then they were recovered and re-suspended in 50 µL phosphate buffer (0.02 M, pH7), and tested against the indicator strain *E. faecium* H3.

Estimation of the bacteriocin molecular weight by Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Schägger (2006), using 4% stacking gel, 10% spacer gel and 16% separating gel. The samples tested were the native supernatant, the concentrated supernatant, the fractions obtained after purification thanks to Sephadex G25, CM Sephadex Cellulose and HPLC. Molecular weight protein standards (Nisin 3.5 kDa and BSA 67 kDa) were used. SDS-PAGE was conducted overnight at 50V, 20 mA. After electrophoresis, the gel was cut vertically into two parts, the part of the gel that contained samples and protein markers was stained with a solution of Coomassie Brilliant Blue R-250 (Merck, Saint Louis, MO), while the remaining part containing only the samples was fixed in a mixture of 20% isopropanol (v/v), 10% acetic acid (v/v) for 1 h, and washed with sterile distilled water overnight and used for detection of antibacterial activity by overlaying with MRS soft agar inoculated with 800 µL of a 18 h culture of the indicator strain *E. faecium* H3 and incubated for 24 h at 30°C.

Mode of action of bacteriocin GHB26

The bacteriocin was tested for a bacteriostatic or bactericidal mode of action against *E. faecium* H3 as follows: 2 ml of concentrated bacteriocin was added to 10 ml growing cells of *E. faecium* H3 in MRS broth medium in early exponential phase (4 h at 30°C). A sample culture of *E. faecium* H3 without bacteriocin was used as a control. Optical density at 600 nm was determined at appropriate intervals.

RESULTS AND DISCUSSION

Detection of inhibitory activity

The strain *Enterococcus* sp. GHB26 inhibited the growth of indicator strain *E. faecium* H3. This antibacterial activity was recorded on buffered MRS broth with a maximum activity level of 1066 AU/ml, which excludes the possible effect of lactic acid. *Enterococcus* sp. GHB26 was found to produce an inhibitory substance against *E. faecium* H3.

Physico-chemical characterization of the inhibitory substance

Effect of enzymes

The antibacterial activity of the cell-free supernatant was sensitive to proteolytic enzymes, except pepsin, which indicated the proteinaceous nature of the active substance. The inhibitory activity was not affected by catalase, indicating that inhibition was not due to hydrogen peroxide production (Table 1). The inhibitory substance produced by *Enterococcus* sp. GHB26 may be considered as a bacteriocin, that we designated bacteriocin GHB26. Klaenhammer (1988) reported that the bacteriocins were sensitive at least for one proteolytic enzyme.

Effect of temperature and pH

The antibacterial activity of the inhibitory agent was

Table 1. Effect of enzyme, temperature, pH and chemical agents on inhibitory activity.

Treatment	Inhibitory activity (AU/ml)
Untreated	1066
Enzymes	
Catalase	1066
Trypsin	0
Pronase E	0
Pepsin	1066
Temperature	
60°C at 60 min	1066
60°C at120 min	1066
80°C at 60 min	1066
80°C at120 min	1066
100°C at 60 min	1066
100°C at120 min	1066
120°C at 20 min	533
pH	
2	1066
4	1066
6	1066
8	1066
10	1066
12	533
Chemical agents	
SDS (1%)	1066
Tween 80 (1%)	1066
Urea (1%)	1066
NaCl (1%)	1066
EDTA (1%)	1066
TritonX-100(1%)	0

stable to heat treatment at 60, 80 and 100°C for 60 and 120 min (1066 AU/ml). However, heating at 120°C reduced the activity to 50% after 20 min (533 AU/ml) (Table 1).This was in line with enterococcal bacteriocins that were reported to be heat stable (Park et al., 2003). The antibacterial activity was maintained stable over the range of pH from 2 to 10 (1066 AU/ml). However, a 50% loss of this activity at pH 12 (533 AU/ml) was noticed (Table 1). Similar results were observed for the bacteriocins produced by *E. faecium* ST5Ha (Todorov et al., 2010) and *E. faecium* GHB21 (Merzoug et al., 2015). The resistance to heat and to a wide pH range is an important characteristic for the application of this bacteriocin GHB26 as food biopreservative.

Effect of chemical agents

Treatment of cell-free supernatant with different chemical agents such as SDS, Urea, NaCl, Tween 80, EDTA did not affect the antibacterial activity of the bacteriocin GHB26, except Triton X100 (Table 1). Von Mollendorff et al. (2006) noticed a total loss of antibacterial activity of the bacteriocin JW11BZ from *Lactobacillus fermentum* after treatment with Triton X-100.

Purification of bacteriocin GHB26

Precipitation by ammonium sulfate

Maximum antibacterial activity was obtained when the cell-free supernatant was precipitated with 60% ammonium sulfate saturation (2132 AU/ml). Similar result was observed by Ogunbanwo et al. (2003) for the bacteriocin produced by *Lactobacillus plantarum* F1.

Sephadex G-25 gel filtration chromatography

Several fractions were collected (Figure 1). Only three

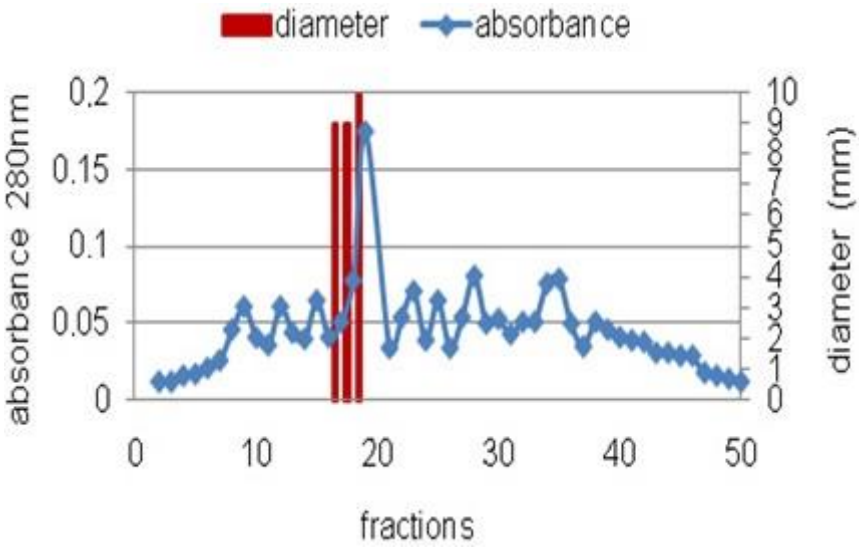

Figure 1. Sephadex G25 chromatogram of the active fraction from precipitation with ammonium sulfate at 60%.

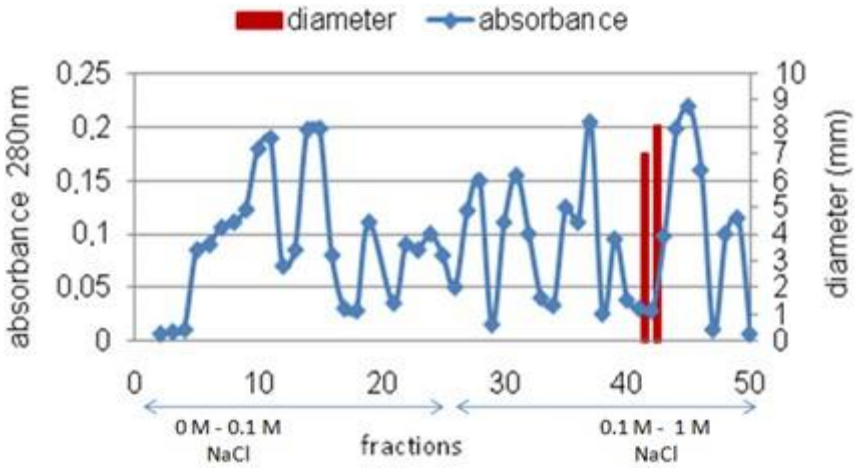

Figure 2. CM-Sephadex cellulose chromatogram of concentrated active fractions from Sephadex G-25 column.

fractions (16-17-18) have shown inhibitory activity against *E. faecium* H3. Sephadex G-25 column can separate proteins ranging in molecular weight from 1000 to 5000 Da. Obtained results suggest that the inhibitory substance has a molecular weight lower than 5000 Da since it was eluted with the elution volume. Similar result was reported by Smaoui et al. (2009) for the bacteriocin BacTN635 produced by *L. plantarum* TN635.

Cation exchange chromatography in a CM- Sephadex Cellulose column

Several fractions were collected (Figure 2). Only two fractions (42-43) have shown inhibitory activity against *E. faecium* H3. The active substance obtained from cation exchange chromatography was not eluted with a NaCl concentration of 0 to 0.1 M, but at a gradient of 0.1 to 1 M. This result shows that the bacteriocin GHB26 is cationic. Vera Pingitore et al. (2007) reported that in a standard protocol, the bacteriocin extract is passed through the cation exchange column; the bacteriocin eluted using a gradient of NaCl from 0.1 to 1 M.

Purification by reverse phase-HPLC (RP-HPLC)

RP-HPLC chromatogram showed a single peak (Figure 3).

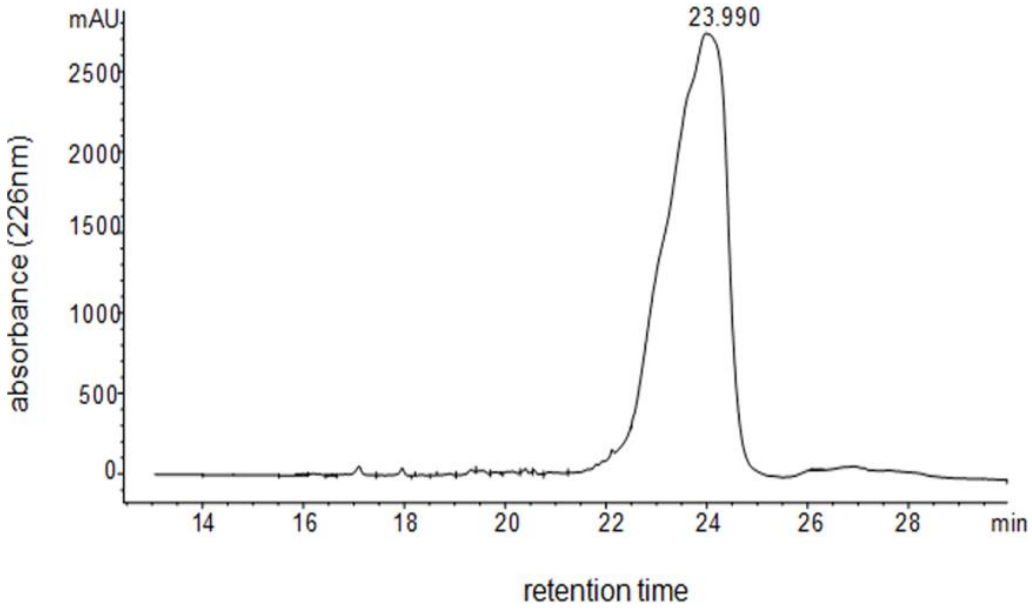

Figure 3. RP-HPLC Chromatogram of concentrated active fractions from CM- Sephadex Cellulose column.

This peak was eluted at 85% of acetonitrile, corresponding to a retention time of 23.99 min. The peak was shown to be active against *E. faecium* H3. This final purification step by RP-HPLC indicates the purity of the bacteriocin GHB26. Earlier reports (Elegado et al., 1997; Srinivasan et al., 2012) have obtained a single peak by RP-HPLC and these authors explain such a result by a high purity of the pediocin AcM from *Pediococcus acidilactici* M and the bacteriocin produced by *Lactobacillus rhamnosus* L34. However, it was observed that the bacteriocin GHB26 was eluted at 85% acetonitrile, which would correspond to an elution of hydrophobic protein (Kawai et al., 1994).

Using nisin as a standard, its elution was recorded to a retention time of 26 min. Verdon (2009) showed that the bacteriocins that have the same size and the same hydrophobicity properties will elute by RP-HPLC at very near time; this is the case of the bacteriocin GHB26 (23.99 min) and nisin (26 min).

SDS-PAGE for molecular weight determination

A Coomassie blue-stained SDS-PAGE gel revealed a diffuse band of the concentrated supernatant (Figure 4a). A comparison of the band on the stained gel with the half gel showing inhibition zones (Figure 4b) indicates that the band contained the active bacteriocin GHB26. In the case of native supernatant, no protein band was detected despite the presence of the inhibition zone. This zone was very close to nisin zone, corresponding to about 3.5 kDa. For the other samples (fractions obtained after purification by Sephadex G25, CM Sephadex cellulose and HPLC) no bands were observed on the stained gel and no zone of inhibition was observed on the native gel. BSA is observed in lanes 1 and 2 of the gel.

SDS-PAGE electrophoresis showed that the bacteriocin GHB26 has a molecular weight close to that of nisin (3.5 kDa).This result confirms what was previously observed by RP-HPLC. The obtained molecular weight of bacteriocin GHB26 averages within the size range of most bacteriocins from enterococcal strains. For example, enterocin 1071B produced by *E. faecalis* BFE 1071 was reported to have a molecular weight of 3.899 kDa (Balla et al., 2000), enterocin MC13 from *E. faecium* MC13 was reported to be 2.148 kDa (Satish Kumar et al., 2011).

Mode of action of bacteriocin GHB26

The optical density of the indicator strain culture remained constant after the addition of the bacteriocin (Figure 5). Hence the mode of action of the bacteriocin GHB26 was considered as bactericidal. Similar results were reported for bacteriocins produced by *E. faecium* LR/ 6 (Kumar et al., 2009) and *Lactobacillus salivarius* SMXD51 (Messaoudi et al., 2012).

Conclusion

Enterococcus sp GHB26 was able to produce an effective bacteriocin with interesting features. The

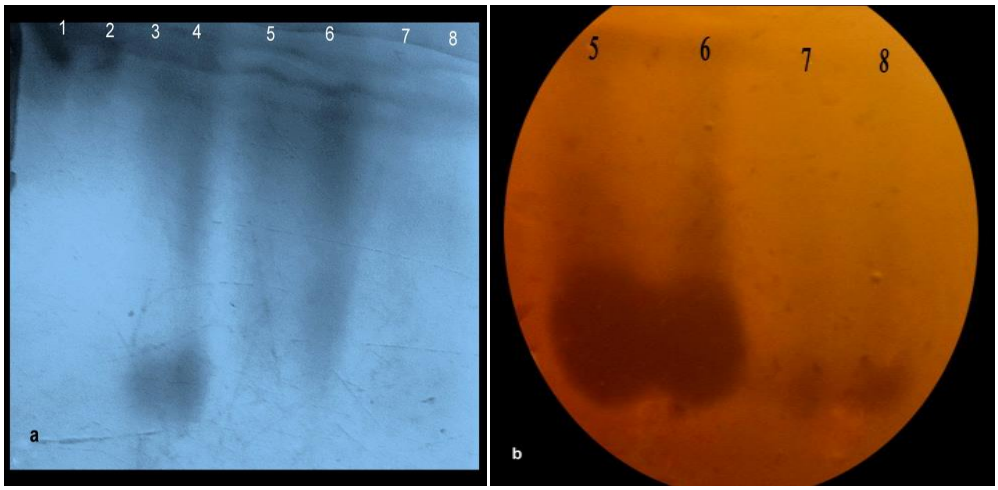

Figure 4. a. SDS-PAGE gel stained with Coomassie blue; b. Bacteriocin activity in native gel. Lane 1: BSA (30 µl), lane 2: BSA (60 µl), lane 3: nisin (30 µl), lane 4: nisin (60 µl), lane 5: concentrated supernatant (30 µl), lane 6: concentrated supernatant (60 µl), lane 7: native supernatant (30 µl), lane 8: native supernatant (60 µl).

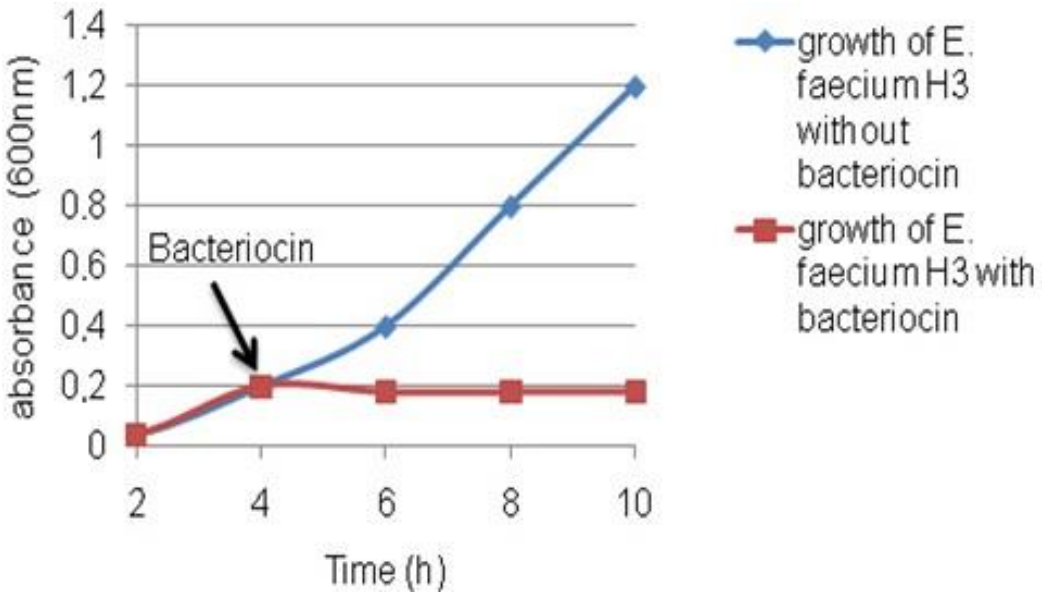

Figure 5. Growth of *Enterococcus faecium* H3 without bacteriocin GHB26 and with bacteriocin GHB26.

bacteriocin GHB26 was heat stable, sensitive to proteolytic enzymes, resistant to different pH values and to several chemical agents. For bacteriocin GHB26 purification, a four-step method including ammonium sulfate precipitation, Sephadex G-25 gel filtration chromatography, cation exchange chromatography on CM-Sephadex cellulose and RP-HPLC allowed the obtention of a pure bacteriocin, with a molecular weight close to 3.5 kDa. These results indicate that the bacteriocin GHB26 could be classified in the class II of bacteriocins. Bacteriocin GHB26 exhibited an inhibitory effect against *E. faecium* a frequent contaminant of milk and grains in our region. Based on these interesting characteristics, bacteriocin GHB26 could be a promising biopreservative in food.

Conflict of Interests

The authors have not declared any conflict of interests.

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