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Full Length Research Paper

Evaluation of the effect of some environmental parameters on the level of bacteriocin activity

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***Bacillus megaterium* 22, a soil isolate, produced a bacteriocin that exhibited a broad range of inhibitory activity against food-spoilage microorganisms including *Salmonella typhimurium* and *Staphylococcus aureus*. The antimicrobial activity peaked at the early stationary phase. De Man Rogosa Shrarpe (MRS) was the best medium for bacteriocin production, where growth of *B. megaterium* 22 for 12 - 18 h at 30°C, pH 6 - 6.5 resulted in maximum inhibitory effect on the pathogenic indicator strains. Supplemen-tation and/or replacement of medium nutrients demonstrated higher values of bacteriocin activity in the presence of 5 - 10% sucrose, 1% beef extract, and under limited aeration. Bacteriocin activity was significantly stimulated at concentrations of up to 3% NaCl, or 1% KCl. Low levels of spices (curry, red and Black pepper) synergistically stimulated the bacteriocin activity, except for garlic and rosemary where higher concentrations (1%) considerably influenced the activity. The bacteriocin was heat stable for 15 min of exposure to a wide range of temperatures, and over a pH range of 2 - 8 after 1 h of expo-sure. The bacteriocin was stable for up to 30 min of exposure to UV light, and when stored at 4°C for 90 days. The activity was inhibited by proteolytic enzymes and tested organic solvents. SDS-PAGE revealed that the apparent molecular weight of the partially purified bacteriocin ranged from 3.496 to 6.512 kDa. Results presented here support the idea that the bacteriocin may propose some industrial advantages that render it as a good natural food bio preservative candidate.**

Key words: *Bacillus megaterium*, antimicrobial activity, bacteriocin, biopreservation, growth media, spices, storage, indicator strains.

INTRODUCTION

Bacteriocins are antimicrobial ribosomally synthesized peptides produced by bacteria that inhibit or kill micro-organisms that are usually, but not always, closely rela-ted to the producer strain (Sánchez-Hidalgo et al., 2008). Also include the original (first report) reference here and not just the latest one. Different species of *Bacillus* pro-duce bacteriocins, and within a species either several different kinds of bacteriocins (Motta and Brandelli, 2008) or bacteriocin-like substances (BLS) (Motta et al., 2008) may be produced with varying modes of action. Bac-teriocins produced by “food grade” lactic acid bacteria (LAB) have long been the focus of exten-sive studies with the perspective of their potential and effective use and application as nontoxic natural food biopreservatives for the food industry and therapeutic agents for gastrointestinal infections (Haugen et al., 2008). *Bacillus* is an interesting genus to investigate for antimicrobial activity since *Bacillus* species produce a diverse array of anti-microbial peptides representing several different basic chemical structures (Bizani and Brandelli, 2002), with a distinct diversity in their inhibitory activities against a va-riety of micro-organisms (Korenblum et al., 2005). Bacteriocins have been studied in different species including: *Bacillus subtilis*, *Bacillus cereus*,

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Bacillus stearothermophilus, *Ba-cillus Licheniformis*, *Bacillus thuringiensis* , and other *Bacillus* species (Pattnaik et al., 2001). The best-charac-terized bacteriocin was thuricin produced by strains of *B. thuringiensis* (Ahren et al., 2003). Other bacteriocins have been reported such as lichenin which was produced by *B. licheniformis* 26-103RA strain (Pattnaik et al., 2001), megacin produced by strains of *B. megaterium* (Lisboa et al., 2006), antilisterial coagulin produced by *B. coagulans* (Le Marrec et al., 2000), and cerein produced by strains of *B. cereus,* (Naclerio et al., 1993). Moreover, some of these bacteriocins have been indicated as po-tential biopreservatives in food systems and beverages, as agents for biological control of phyto-pathogens (Bais et al., 2004), and as antibiotic precur- sors (Zuber et al., 1993). However, limited data exists on the applications of bacteriocins from *Bacillus* spp and in particular from members of *B. megaterium*. In the recent time, it was reported that some strains of *B. megaterium* produced several bacteriocins effective against other strains of *B. megaterium* (Holland and Roberts, 1964; Von Tersch and Carlton, 1983). But as far as we could determine, the antimicrobial properties of *B. megaterium* has not been fully explored for use as biopreservative, largely due to limited production techniques (Reddy et al., 1984). Hence, the search for different bacilli strains pro-ducing new antimicrobial agents with wider spectrum of activity and compatibility with different food systems is still desirable for many processing systems. The biopreservation capacity of a bacteriocin could be achieved either by using a bacteriocin-producing starter culture or by applying the bacteriocin itself as a food additive.

Therefore, a thorough study of the essential parameters affecting the inhibitory activity (Zala´n et al., 2005), followed by optimization of production that is usually dependent on multiple strain- specific factors (Leal-Sa´nchez et al., 2002) is necessarily required for introducing a bac-teriocin into foods as a potent biopreservative. This raised the need and was the driving force for our present investigation, in which we report on a bacteriocin pro-duced by *B. megaterium* isolated from soils of local terri-tory in Alexandria, Egypt. In this approach, the effect of some environmental parameters on the level of bacte-riocin activity is evaluated. We ascribe the influence of cultural conditions such as nutrients, nutrient concentra-tion, nutrient combinations/interactions, aeration, and other physical factors including heat, UV, pH, storage conditions for the purpose of obtaining better and stable bacteriocin activity.

MATERIALS AND METHODS

Bacterial strains, inoculum preparation, and cultural conditions: The strain of *B. megaterium* isolated from soils of the local territory (native lands) in Alexandria, Egypt was identified according to on the basis of its cultural, the morphological and biochemical properties, physiological, biochemical characteristics, and carbohydrate fermentation tests (Schillinger and Lücke, 1987). This was followed by partial 16S rRNA analysis (Maidak et al., 1999) conducted at the German culture collection of microorganisms and cell cultures DSMZ (Deutch Sammlung von Mikroorganismen und Zelkulturen GmbH). The strain was described as *B. megaterium* 22. The test microorganisms used in our study include: *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Salmonella* para-*typhimurium* A, *Salmonella* para-*typhimurium* B and *Enterococcus faecalis*, *Streptococcus pyogenes*, and *Staphylococcus aureus* were supplied from the Microbiology Laboratory at the Faculty of Medicine, Alexandria University, and were used to determine the antimicrobial spectrum of bacteriocin. *S. typhimurium* and *S. aureus* were chosen as the indicator strains in all the antimicrobial assays. Bacterial strains were propagated in MRS broth (De Man Rogosa Sharpe) (Biolife Italiana S.V.L), kept frozen in 20% (v/v) glycerol at -20^{o}C until needed and subcultured twice before use. Unless otherwise stated, test strain inoculum (1% v/v) consisted of cellular suspensions from 12 - 18 h MRS cultures, incubated at 30^{o}C, adjusted to an absorbance (600 nm) of 1 - 1.2, while indicator strains inoculum were prepared similarly but monitored spectrophotometrically till an O.D.$_{600}$ corresponding to 10^{5} CFU/ml was reached (Naclerio et al., 1993).

Bacteriocin preparation

The cells of *B. megaterium* 22 were grown as 2% in MRS broth at 30^{o}C, collected after 12 h at the early stationary phase by centrifugation at 10,000 x g for 20 min at 4^{o}C. The cell free supernatant (CFS) was passed through membrane filters (Renner GMBH D-67125/ Germany) with a pore diameter of 0.2 m, and stored in the refrigerator for a maximum period of two weeks and periodically tested for the bacteriocin titer before being renewed.

Antimicrobial spectrum

The agar well diffusion (AWD) assay was used to determine the antimicrobial spectrum of the test strain producing bacteriocin above mentioned (Lasta et al., 2008). Diameters of inhibition zones were scored (Korenblum et al., 2005).

Detection of bacteriocin activity during growth (growth kinetics)

MRS broth was inoculated with 2% (v/v) of an overnight pre-culture of the test strain, incubated at 30^{o}C, where changes in O.D.$_{600}$ were recorded every 3 h. The growth kinetics experiment (Vinderola et al., 2002) was performed with a minor modification. The indicator strains (10^{5} CFU/ml) were grown at 30^{o}C in MRS broth in the presence of the test strain CFS. Optical density measurements (ODM) were recorded every 3 h for 12 - 18 h. The bacteriocin activity was expressed by the percentage of growth reduction to the indicator strains and determined from the ratio between the optical densities of the treated cultures and untreated ones (the indicator strains without the CFS). This ODM method was used in all the antimicrobial assays

The influence of growth conditions on bacteriocin activity

The effect of growth media, incubation temperature, initial pH, and aeration on bacteriocin activity was carried out as follows: *B. megaterium* 22 was grown aerobically at 30 and 37^{o}C in five different me-

Table 1. Influence of inorganic salts and spices on the activity of *B.megaterium* 22 bacteriocin. Results are % of mean values of activity ± standard deviations (n=3).

Inorganic salt	Indicator strains	Concentration (%)			
		0.0	0.5	1.0	3.0
NaCl	*S. typhimurium*	87.02.1 ± 0.3	86.8 ± 0.3	95.7 ± 1.3	96.7 ± 1.5
	S. aureus	88.1 ± 1.2	88 ± 0.3	96.7 ± 1.2	93.6 ± 1.3
KCl	*S. typhimurium*	87.02.1 ± 0.3	83.9 ± 0.6	92.7 ± 0.6	91 ± 1.5
	S. aureus	88.1 ± 1.2	83.5 ± 1.5	92.2 ± 0.6	91.5 ± 1.2
$MnCl_2$	*S. typhimurium*	87.02.1 ± 0.3	79 ± 1.0	15 ± 0.8	6 ± 0.4
	S. aureus	88.1 ± 1.2	76 ± 0.9	16 ± 0.7	3 ± 0.2
Curry	*S. typhimurium*	87.02.1 ± 0.3	93 ± 0.2	53 ± 0.1	46 ± 0.1
	S. aureus	88.1 ± 1.2	94 ± 0.2	54 ± 0.1	46 ± 0.1
Red pepper	*S. typhimurium*	87.02.1 ± 0.3	84 ± 0.4	82 ± 0.4	84 ± 0.4
	S. aureus	88.1 ± 1.2	83 ± 0.4	82 ± 0.4	84 ± 0.4
Black pepper	*S. typhimurium*	87.02.1 ± 0.3	79 ± 0.1	69 ± 0.2	64 ± 0.2
	S. aureus	88.1 ± 1.2	79 ± 0.2	69 ± 0.2	63 ± 0.1
Bastermy	*S. typhimurium*	87.02.1 ± 0.3	44 ± 0.3	-103 ± 8.4	-114 ± 4.8
	S. aureus	88.1 ± 1.2	43 ± 0.2	-92 ± 5.3	-163 ± 6.4
Garlic	*S. typhimurium*	87.02.1 ± 0.3	94 ± 0.3	98 ± 8.4	77 ± 4.8
	S. aureus	88.1 ± 1.2	89 ± 0.2	96 ± 0.8	75 ± 6.4
Rosemary	*S. typhimurium*	87.02.1 ± 0.3	92 ± 2.0	50 ± 0.4	34 ± 0.3
	S. aureus	88.1 ± 1.2	96 ± 1.2	42 ± 0.3	23 ± 0.2
Paprika	*S. typhimurium*	87.02.1 ± 0.3	28 ± 0.2	-33 ± 2.4	-74 ± 1.3
	S. aureus	88.1 ± 1.2	16 ± 0.7	-42 ± 0.3	-53 ± 1.2

dia, MRS (De man Rogosa Sharpe) broth, Brain Heart Infusion (BHI; Merck, Darmstadt, Germany) broth, M17 (Difco Laboratories, Detroit, MI) broth, whey and molasses (2% v/v). Aliquots of MRS broth were adjusted with 1 N HCl or 1 N NaOH to pH values of 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 (Ougnbanwo et al., 2003a), autoclaved, and inoculated with the test strain. The effect of aeration conditions on bacteriocin activity was studied by varying the volume of the growth medium where aliquots of 10, 20, 40 and 50 ml were inoculated with the test strain. All preparations with different conditions were propagated under the same conditions, and assayed for bacteriocin activity as described above.

Influence of medium components on bacteriocin activity

The effect of medium ingredients on bacteriocin production was evaluated using modified MRS broth. The supplements studied were: 2 and 4% of monosaccharide (galactose) and disaccharide (fructose, lactose, maltose, and sucrose) sugars respectively, after replacing the medium sugar glucose (Ogunbanwo et al., 2003b). Subsequently the effect of higher sucrose concentrations, up to 10% (w/v) was studied. The effect of nitrogen sources on bacteriocin activity was also evaluated using (/lit): 4 g ammonium acetate, 4 g ammonium chloride, 4 g ammonium nitrate, 4 g ammonium sulfate, 4 g arginine, 10 g beef extract, 4 g sodium nitrate, 10 g tryptone, and 5 g yeast extract. The tested combinations of nitrogen (1.5 and 0.5%) included: beef extract plus yeast extract, beef extract plus tryptone, beef extract plus ammonium chloride, beef extract plus arginine. The activity of the CFS from each culture condition was assayed.

The influence of some inorganic salts and spices on bacteriocin activity

The inhibitory effect of inorganic salts (KCl, $MnCl_2$, and NaCl) on bacteriocin activity was tested (Karao lu et al., 2003) by mixing concentrations of 0.5, 1, and 3% of each salt to the CFS preparations for 2 h. Spices (Table 1) used as local food additives were also studied for their possibility of influencing the effectiveness of the bacteriocin activity (Verluyten et al., 2004). Each spice was dissolved in 10 ml of sterile warm distilled water, vortexed for 5 min, followed by centrifugation, filter-sterilization, and mixed for 2 h with the CFSs to get a final concentration of 0.5, 1, and 3% (v/v). Salts and spices-treated preparations were assayed for antimicrobial activity as previously described.

The influence of heat, UV, pH, storage on bacteriocin activity

For heat treatment, the CFS preparations were heated for 15 min at 30, 40, 50, 60, 70, 80, 90, 100 and 121°C (Mota et al., 2004). Similarly, sterile petri dishes containing 10 ml aliquots of crude bacteriocin preparations were exposed from 15 to 90 min to UV irradiation (Philips bulb, wave length 340 nm, 220 - 240 V, 50 Hz,) situated 30 cm distance from the Petri dishes (Wanda and Bonita, 1991; Ogunbanwo et al., 2003a). The effect of pH on activity was tested by adjusting the CFSs to pH values from 2 to 12 (at one unit increments) with sterile 1 N NaOH or HCl (Albano et al., 2007). Samples were incubated at ambient temperature (~25^{O}C) for 1 h. The crude bacteriocin was stored at –20 and 4ëC for different intervals of time (30, 45, and 60 days). The activity in all prepara-

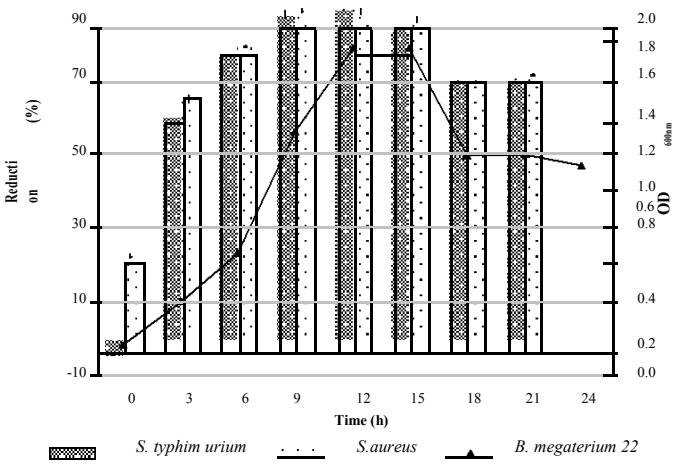

Figure 1. Bacterial growth (▲) and bacteriocin activity of *B. megaterium* 22. The strain was grown aerobically in MRS broth at optimal conditions of temperature (30°C), for 24 h. Activity of bacteriocin preparations against *S. typhimurium* (▩) and *S. aureus* (⁘) was expressed as % reduction of growth. Each point represents the mean ± S.E.M. of three independent experiments.

tions and aliquots was determined as previously mentioned.

The influence of proteolytic, non-proteolytic enzymes and solvents on bacteriocin activity

Proteolytic enzymes (Oxford laboratory reagents) including papain, pepsin, trypsin, and non-proteolytic enzymes (lipase and - amylase) were dissolved in 0.05 M sodium phosphate (pH 7.0), 0.002 M HCl (pH 7), 40 mM Tris-HCl (pH 8.2), 0.1 M potassium phosphate (pH 6.0), and 0.1 M potassium phosphate (pH 7.0) respectively to a final concentration of 1 and 2 mg/ml. Enzyme solutions were filter sterilized, mixed with aliquots of filter sterilized CFSs of the test strain, incubated at 30^{O}C for 2 h, subsequently heated in boiling water for 5 min to inactivate the enzymes (Bizani and Brandelli, 2002), and assayed for antimicrobial activity. The sensitivity of freeze dried bacteriocin preparations to organic solvents such as acetone, chloroform, ethyl alcohol, hexane, and methanol was investigated (Todorov et al., 2006) by being dissolved in each organic solvent to a final concentration of 10 mg/ml. Samples were incubated at 30°C for 1 h, solvents were removed by evaporation, and dried residues from the organic phase were re-suspended in sterile MRS broth (Ten Brink et al., 1994) and assayed for antimicrobial activity

Partial purification and molecular weight determination

The test strain was grown in MRS broth for 10 h at 30^{O} C. Cells were harvested by centrifugation at 10,000 x g for 20 min at 4^{O}C, after which the bacteriocin was precipitated from the CFS with 45% saturated ammonium sulfate (Akyptis et al., 1998). After 4 h of stirring at room temperature, proteins were removed by centrifugation, and dissolved in 20 mM tris-HCl buffer (pH 7). The mixture was then dialysed using a Spectra/Por membrane tubings (Spectrum laboratories Inc., CA, USA) of 12 kDa cut off against 2 L of distilled water for 24 h at 4°C with at least 3 changes. The dialyzed preparation was lyophilized resulting in the formation of a dry precipitated residue referred to as the partially purified bacteriocin. Its molecular weight was estimated as described by Sambrook and Russell (2001) using discontinuous SDS-PAGE (11%) performed using a double slab electrophoresis cell (Cleaver scientific Ltd). The molecular mass was calculated by comparison with the mobility of standard markers (Bio-RAD, Germany) ranging from 90 - 1.434 kDa.

Statistical analysis

Data were expressed as mean ± standard deviation. Statistical significance was determined using one-way analysis of variance on the replicates, where a p-value of 0.05 was considered significant.

RESULTS

Antimicrobial spectrum

The CFS of *B. megaterium* 22 contained an antimicrobial compound with a wide spectrum active against representtatives of three Gram -positive and Gram- negative pathogenic strains (*E. coli*, *K. pneumoniae* , *S. aureus*, and *S. typhimurium*). The average diameter of inhibition zones as determined by the AWD method ranged from 0.5 - 5 mm in size (data not shown). It is worth mentioning that the producing strain was not inhibited by its own bacteriocin.

Growth kinetics

Figure 1 depicts high bacteriocin activity against *S. aureus* at zero time, and during the first three hours of incubation against both indicator strains (at least 60% growth reduction). Maximal antibacterial activity was achieved at the early stationary phase after 12 - 15 h, after which the growth started to decline gradually as determined by culture turbidity and bacteriocin activity, where no activity was recorded after 24 h of experimentation.

The influence of growth conditions on bacteriocin activity

In general, the best results corresponding to the highest inhibitory effect on the indicator strains were obtained using MRS broth after 12 - 18 h of incubation at 30^{O}C, followed by M17, finally by using whey (Figure 2). Good antimicrobial activity was recorded in the presence of 2% (w/v) molasses after 12 - 18 h of incubation. BHI medium was not suitable for bacteriocin production. *B. megaterium* bacteriocin exhibited lower activity against the indicator strains upon increasing the incubation temperature when using MRS broth as the growth medium. However, growth particularly in whey at 37^{O}C resulted in an excep-

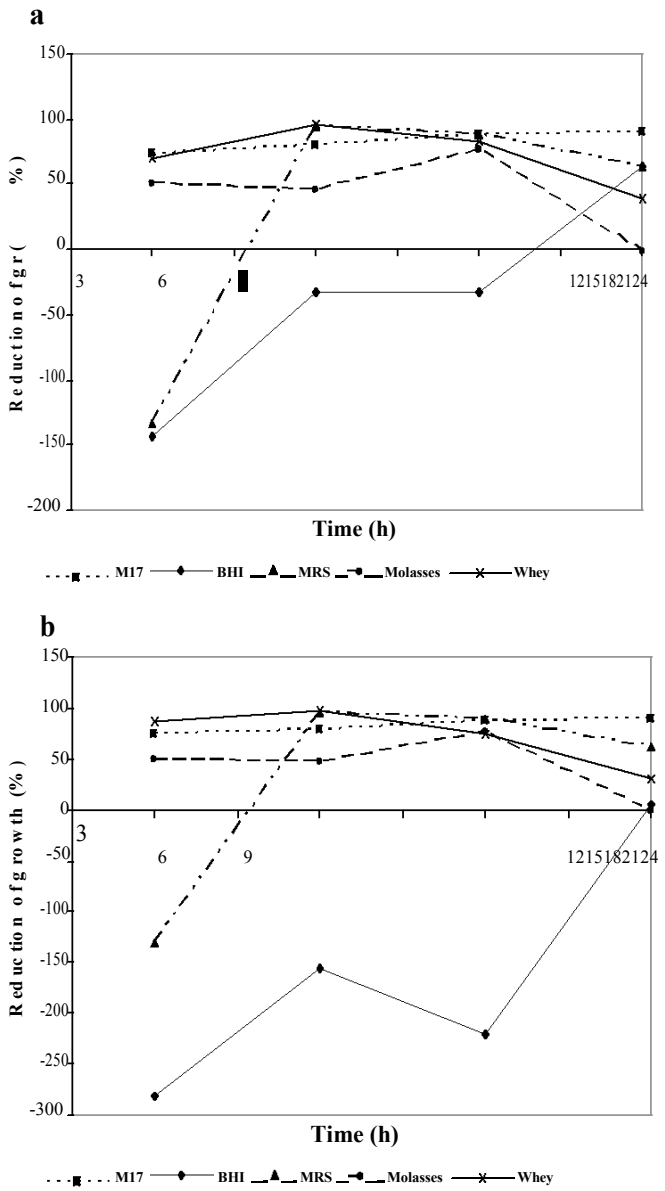

Figure 2. Influence of growth media on the bacteriocin production of *B. megaterium* 22 against the indicator strains *S. typhimurium* (**a**) and *S. aureus* (**b**) when grown in MRS broth at 30°C, pH: 6.2 – 6.5. Results are expressed as % of mean values of activity (n=3) ± standard deviations.

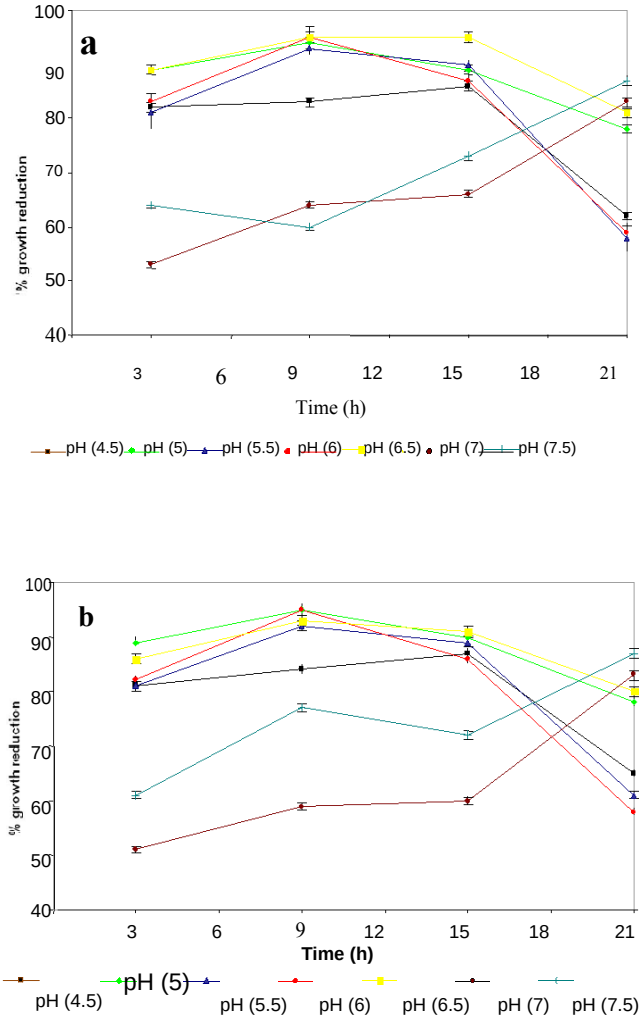

Figure 3. Effect of the initial pH on bacteriocin production of *B. megaterium* 22 against *S. typhimurium* (**a**) and *S. aureus* (**b**) when grown in MRS broth at 30ëC. Results are expressed as % of mean values of activity (n=3) ± standard deviations.

tion to this trend (data not shown). pH 6 - 6.5 fostered the maximum bacteriocin activity (almost 95% growth reduction) against the indicator strains, followed by pH 5, after growth for 12 - 18 h in MRS broth (Figure 3). The acidic and the alkaline pH values 4.5 and 7.5 had significant adverse effects (P>0.05) on decreasing the bacteriocin activity. The strain proved to produce bacteriocin under limited or reduced aeration in the medium, where the highest bacteriocin activity (91 - 92% growth reduction) was attained in presence of 40 ml of the growth medium compared to the activity in presence of lesser volumes (data not shown).

The influence of medium components on bacteriocin activity

The best alternative sugar to glucose that yielded high bacteriocin activity was sucrose followed by maltose, fructose, then finally lactose and galactose after 15 - 18 h of incubation at 30^{o}C (Figure 4). Based on displaying the most significant effect on bacteriocin activity, different

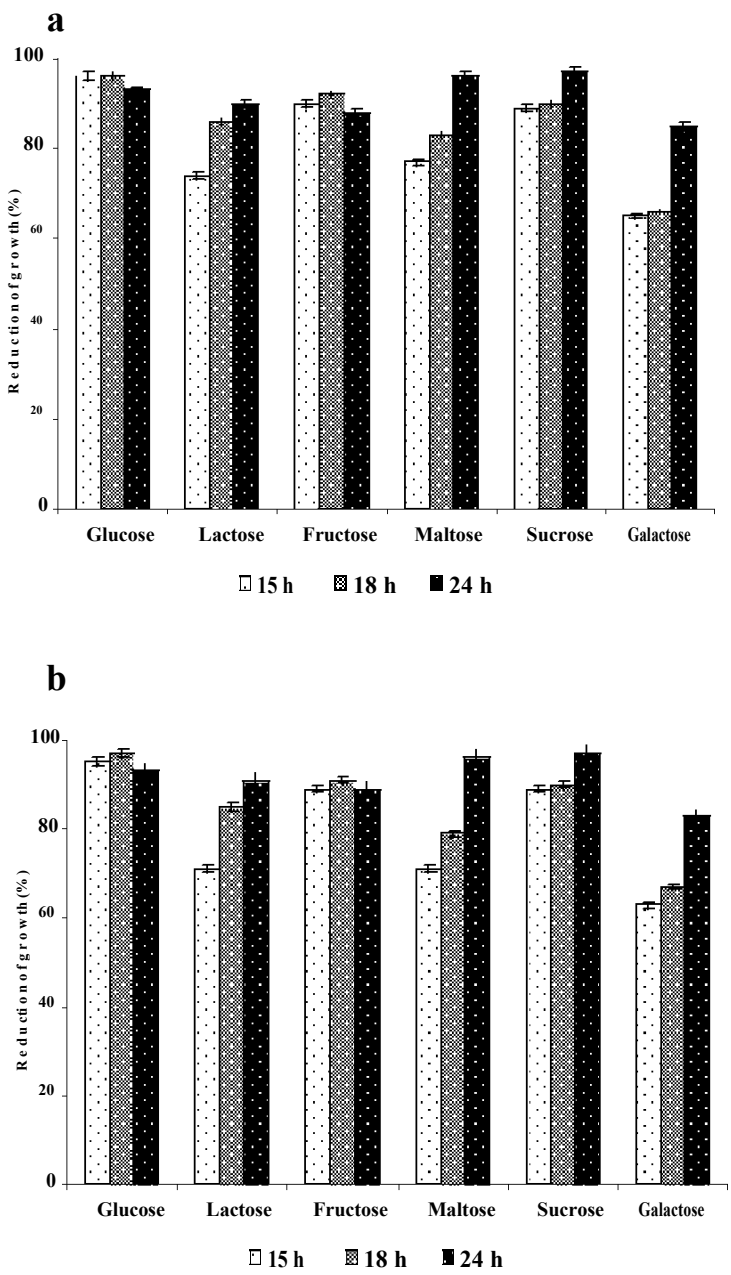

Figure 4. Influence of different carbon sources (g/l) on the bacteriocin production of *B. megaterium* 22 against the indicator strains *S. typhimurium* (**a**) and *S. aureus* (**b**) when grown in MRS broth at 30°C, pH: 6.5. Results are expressed as % of mean values of activity (n=3) ± standard deviations.

concentrations of sucrose were tested. Increasing the concentration to 5, 7, and 10% corresponded to an outstanding level of bacteriocin activity (approximately 100% growth reduction) after 12 - 15 h of incubation (Figure 5). Alteration in the nitrogen source of the growth medium had a significant visible effect (P<0.001) on promoting the bacteriocin antimicrobial activity. The highest inhibitory effect on the indicator strains were obtained after 15 - 24 h of growth in MRS broth supplemented with beef extract (1.0%) as the sole nitrogen source. No appreciable acti-

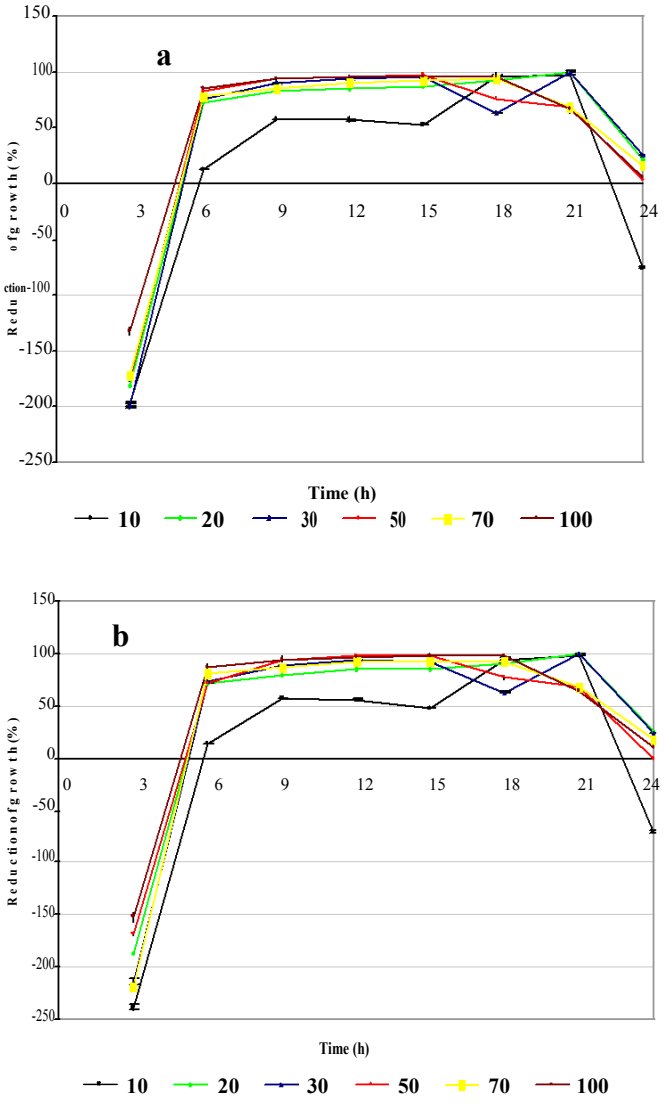

Figure 5. Effect of different sucrose concentrations (g/l) on the bacteriocin production of *B. megaterium* 22 against *S. typhimurium* (**a**) and *S. aureus* (**b**) when grown in MRS broth at 30°C, pH: 6.5.

vity was detected in presence of yeast extract (0.5%) or the rest of the nitrogen sources tested (data not shown). However, yeast extract and beef extract were the favourred combination of organic nitrogen compounds correlated with almost 98% growth reduction to the indicator strains (data not shown).

The influence of inorganic salts and spices on bacteriocin activity

Statistically, high level bacteriocin activity was determined in NaCl concentrations up to 3% (Table 1). Using a concentration of 1% KCl yielded high bacteriocin activity (92.7% growth reduction), whereas concentration of 3% resulted in an opposite behavior. On the other hand, the bacteriocin activity declined significantly (P>0.05) upon treating the CFSs with concentrations of $MnCl_2$ above 0.5%. Spices used in this experiment (curry, red pepper, black pepper, and bastermy sheath) in concentrations ranging from 0.5 - 3% affected the bacteriocin activity in different patterns. The addition of 0.5% curry to the bacteriocin preparations significantly improved the activity against the indicator strains (up to 94% reduction of growth) compared to higher concentrations used (1 or 3%), where a sharp decline in activity was noticed. Concentration of 3% of red pepper had a less pronounced effect on decreasing the bacteriocin activity compared to similar concentration of black pepper. Low values of growth reduction percentages (43 - 44%) were recorded upon treatment with 0.5% of bastermy sheath, and hardly any measurable antagonistic activity was detected when higher concentration was used. Conversely, the addition of either 1% garlic or rosemary to the bacteriocin preparations resulted in a significant activity stimulation, where 93 - 96% growth reduction to the indicator strains was observed. Paprika had the most profound negative effect on the bacteriocin activity, which was more than halfed compared to that of the untreated preparations.

The influence of heat, UV, pH, and storage

Table 2 summarizes the results obtained for different physical treatments of *B. megaterium* 22 CFSs. The antimicrobial substance was stable for 15 min of exposure to use all temperatures, but lost its activity after being autoclaved at 121^{o}C. The bacteriocin maintained its stability up to 30 min of exposure to UV light, however exposure to longer periods resulted in significant decrease in growth reduction values to almost half. UV-treated CFSs showed an overall high antagonistic activity against *S. typhimurium* compared to *S. aureus*. The bacteriocin was active over a pH range of 2 - 8 after 1 h of exposure, but was totally inhibited in the alkaline range. The bacteriocin was resistant to cooling storage for 90 days, where maximum growth reduction of the indicator strains was recorded (88%). Freezing storage (-20^{o}C) negatively influenced the bacteriocin activity after 30 days of exposure, where weak to moderate growth reduction was detected against *S. typhimurium* and *S. aureus* (35 and 65% respectively). The bacteriocin was markedly distorted upon prolonged freezing storage, where no detectable activity was recorded.

Sensitivity to proteolytic and other enzymes, and solvents

The antimicrobial activity against the indicator strains was

Table 2. Effect of thermal, UV light, pH, and storage temperature treatments on bacteriocin activity against (a) S. *typhimurium* and (b) S. *aureus*. Results are expressed as % of mean values of growth reduction (n=3).

Treatment	Growth reduction (%)	
Temperature	**a**	**b**
0ëC/15 min	70	83
30ëC/15 min	70	87
40ëC/15 min	68	85
50ëC/15 min	75	78
60ëC/15 min	78	79
70ëC/15 min	71	81
80ëC/15 min	73	80
90ëC/15 min	69	81
100ëC/15 min	71	81
121ëC/15 min	-168	-284
Exposure to UV light		
15 min	79.5	11
30 min	72	22.5
60 min	31.5	21
90 min	26.5	7
pH		
2	56.2	38.1
3	36.8	44.79
4	56.2	54.7
5	56.9	54.0
6	63.9	23.02
7	26	54.7
8	33	48.0
9	-34	-95.4
10	-39	-65.6
11	-400	-41.1
12	-400	-66.2
Storage at 4^{o}C		
30	79	88
45	76	74
90	77	76
Storage at -20^{o}C		
30	35	65
45	5	2
90	0	-1

sensitive to the tested concentrations of proteolytic enzymes (Table 3) except for the non-proteolytic enzymes amylase and lipase when used at low concentrations (1 mg/ml). Treatment with organic solvents (acetone, chloroform, ethanol, hexane, and methanol) led to total inactivation of the antimicrobial substance produced by *B. megaterium* 22 strain (data not shown).

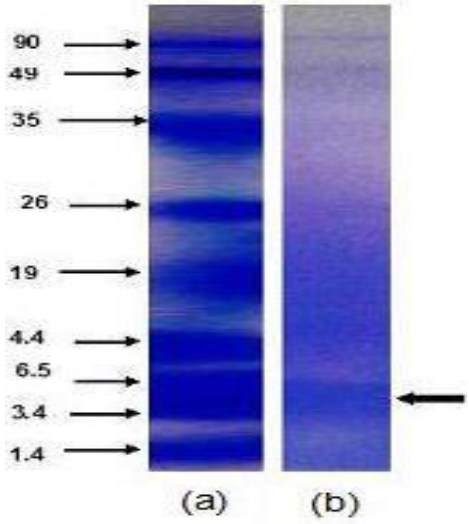

Figure 6. SDS-PAGE electrophoresis of the partially purified bacteriocin of *B. megaterium* 22. Lane **a**: Coomassie Brilliant blue-stained gel with small and large molecular weights of standard markers, lane **b**: Single band of partially-purified bacteriocin. Markers from top to bottom included: Bovine serum albumin (*E. coli*), ovalbumin (chicken egg), carbonic anhydrase (bovine erythrocytes), -lactoglobilin (bovine milk), lyzozyme (chicken egg white), -lactalbumin, aprotinin, insulin drain oxidized, and bacitracin. Sizes on the left are indicated in kDa.

The determination of bacteriocin molecular weight

SDS-PAGE separation indicated that the bacteriocin peptide size ranged from 3.496 to 6.512 kDa (Figure 6).

DISCUSSION

A bacteriocin-producing bacterium was isolated from soil samples of native lands of Alexandria, Egypt. The strain was identified according to biochemical characteristics and by partial 16S rRNA analysis and it was described as *B. megaterium* 22. The maximum production and antibacterial activity of the bacteriocin against the indicator strains *S. typhimurium* and *S. aureus* was found to be at the early stationary phase, after 12 h of growth in MRS broth indicating that the antimicrobial peptide is a secondary metabolite (Lisboa et al., 2006), a character also confirmed by its low molecular weight (Abada, 2008). Naclerio et al. (1993) similarly reported that the production and activity of cerein produced by *B. cereus* was recorded at the stationary growth. Conversely, Cherif et al., (2001) reported that thuricin 7 was produced by species of *B. thuringiensis*, and was expressed in the expo-

Table 3. Effect of enzyme treatment and concentration on bacteriocin activity against (a) S. *typhimurium* and (b) S. *aureus*. Results are expressed as % of mean values of growth reduction.

Enzyme concentration	1 mg/ml		2 mg/ml	
	Growth reduction (%)			
Enzyme	**a**	**b**	**a**	**b**
Papain	-335 ± 47	-212 ± 17	-101.3 ± 19.9	-115 ± 90
Pepsin	-28 ± 11	-85 ± 13	-107.0 ± 13.1	-123 ± 50
Trypsin	-259 ± 94	-154 ± 17	-109.5 ± 7.1	-120 ± 40
-amylase	34 ± 11	11 ± 20	-118.1 ± 0.7	-119 ± 0.3
Lipase	27 ± 80	13 ± 20	-122.8 ± 7.6	-125 ± 80

(n=3) ± standard deviations.

nential growth phase. *B. megaterium* 22 bacteriocin exhibited a wide antimicrobial spectrum, and was capable of inhibiting the growth of some tested microorganisms both Gram-positive and Gram-negative. Korenblum et al. (2005) described a fairly similar result in 90% of their isolated bacilli strains. According to Biswas et al. (1991), modification of cultivation media nutrients should be considered for maximal production of bacteriocin that may have a potential use as food biopreservatives. Hence, diverse set of experiments were designed to deduce the best available cultural condition(s) which could stimulate the activity of *B. megaterium* 22 bacteriocin. Although bacteriocin production is often performed in complex media, which promote abundant growth and relatively high bacteriocin levels, it seemed more economical to test the use of some other media to determine the influence of their components on bacteriocin activity. Our results show that the best medium for bacteriocin activity was MRS broth, whereas low production levels were recorded in BHI broth, molasses, and whey, which suggests that specific nutrients are required for bacteriocin production. This result was consistent with that found by De Kwaadsteniet et al. (2005) where the highest activity of bacteriocin ST15 produced by *E. mundtii* ST15 was recorded after 14 h of growth in MRS broth at 30ëC. Bizani and Brandelli (2002) deduced that the relationship between growth and specific production rates, as a function of the temperature, showed different kinetics of production, where bacteriocin production from *B. cereus* 8A, was higher at 30°C than at 25°C and at 37°C. This observation was inagreement with our results, where the bacteriocin activity against the indicator strains was in general more apparent at 30 than at 37ëC. The maximum value of bacteriocin activity (95% growth reduction) was recorded when *B. megaterium* 22 was grown for 12 h in MRS broth adjusted to initial pH value of 6.5, while lower levels of activity (45 - 65% growth reduction) were recorded at initial pH of 4.5. This was in accordance with observations of Todorov et al. (2006) and Todorov and Dicks (2007), where pH 5.0 and 4.5 repressed the activity of bacHV219 and bacST712BZ respectively. Variation in supplementation of medium/concentration of constituents had a positive influence on bacteriocin activity (Ogunbanwo et al., 2003a), where MRS broth supplemented with 2% sucrose released the best result and the highest inhibitory activity against the indicator strains. The significance of this finding lies in the low cost of sucrose compared to glucose, which could be employed as an economic ingredient in bacteriocin production media. Todorov et al. (2006) reported that yeast extract is the most effective organic nitrogen compound for bacHV219 production. Surprisingly, this finding was not in coherent with our data, where beef extract was the favoured nitrogen source and resulted in accelerating the activity of *B. megaterium* 19 bacteriocin. The bacteriocin was produced under reduced oxygen level in the me-dium, a finding supported by the results of Jurgen et al. (2003) which indicated that oxidative stress caused a higher level of bacteriocin production from *L. curvatus* LTH 1174. The interaction between some food ingre-dients such as inorganic salts and spices with the determined bacteriocin was studied. Concentrations of NaCl and KCl up to 3% stimulated the bacteriocin activity, whereas concentrations of $MgCl_2$ above 0.5% exerted the opposite effect. This could be reasoned to the ability of monovalent cations to mediate the adsorption of bacte-riocin to the indicator strain; while the divalent ions may mask such effect. Moreover, in some cases osmotic stresses appear to favour the bacteriocin release. The response of bacteriocin activity towards the inorganic salts may suggest their synergistic effect when added with specific concentrations in foods. Leroy and De Vuyst (1999) reported that NaCl and $NaN0_2$ can inhibit the bacteriocinogenic effect of bacteriocin-producing cultures in a meat system. Spices and herbs are used for imparting desirable sensory properties to fermented food products (Verluyten et al., 2004), and hence it was important to consider their synergistic effects they may impart

on bacteriocin activity in case they were incorporated with bacteriocinogenic cultures in foods. In general, low concentrations of spices used (curry, red pepper, black pepper) were proportional to the bacteriocin activity. Hugas et al. (2002) indicated that sakacin K production by *L. sakei* CTC 494 was not affected by the addition of 0.4% of black pepper, indicating that small amounts of additives should be preferably used in some foods to promote the activity of the bacteriocins applied. Verluyten et al. (2004) pointed out that combination of spices together with bacteriocins may enable efficient syner-gism, rendering pathogens susceptible to the combined action of bacteriocin and the spices. To an extent, this fact was not reinforced by our result where the bacter-iocin activity was almost halved when mixed with 0.5% of bastermy sheath although it contains more than one spice with different amounts according to different pro-ducers (such as, garlic, salt, chili, feunogreek and pepper etc.). However, this result might be explained on the basis of presence of an unidentified compound that may have led to the inhibition of bacteriocin activity. In con-trast, 1% garlic powder had a significant bactericidal action towards the indicator strains, resulting in 98% growth reduction, while 3% of garlic did not severely decrease the bacteriocin activity. This latter concentration was higher than the inhibitory concentration to lactobacilli as recorded by Gonza´lez-Fandos et al. (1996), which indicates a possible synergism between nisin and garlic extract (Singh et al., 2001). On the other hand, 0.5% rosemary seemed to positively influence the bacteriocin activity, which was in contrast to observations by Verluyten et al. (2004) . The largest negative effect on growth of the indicator strains was ascribed to 0.5% of paprika probably due to interference with production as reported by Verluyten et al. (2004). These results could be of industrial significance, as the bacteriocinogenic strain may be qualified for use in various food product applications based on its ability to stabilize the sensory quality and extend the shelf life of foods synergistically with spices included in the manufacture. Heat treatment of *B. megaterium* 22 bacteriocin expressed an interesting feature of heat stability at wide range of temperatures. A close finding to our result was that of bacteriocin produced by *Lactobacillus CA44* and *L. lactis* subsp. *cremoris* CTC 204 as reported by Vinod et al. (2006) and Bromberg et al. (2005) respectively. The heat stability data may impart another industrial advantage to our producing strain in view of the potential use of its bacteriocin as a food additive in procedures of food preparation involving a heating step, and hence its potential effectiveness against psychrophile, thermophile and mesophile foodborne pathogens. The activity of *B. megaterium* 22 bacteriocin was pH dependent and was stable between pH 2 and 8 at 30^{O}C. A similar behaviour has been reported for pediocin F, which was found to be stable over a wide pH range between 3 and 9 (Özlem et al., 1997). Our pH data propose an additional benefit and a possible advantage to the bacteriocin in food industry, and suggest its effective applicability against molds, yeast, acetobacter, and other deteriorating food microorganisms in neutral and acidic foods. The preservation capacity of the bacteriocin in terms of the period and temperature of storage was quite interesting, as it maintained full stability and was equally active against the two indicator strains after 90 days of storage at 4ëC, besides its partial stability up to only 30 days at -20^{O}C, indicating that cold temperature may be the most appropriate medium of preservation. The protein status of the tested bacteriocin was confirmed by results of UV light, where it demonstrated high resistance to UV exposure for up to 60 min with no change of protein nature or function (Ogunbanwo et al., 2003a). *B. megaterium* 22 bacteriocin was typical to LAB in respect of its sensitivity to trypsin (Jack et al., 1995), as well as to papain and pepsin. This resistance can be explained on the basis of the presence of unusual amino acids in the bacteriocin structure, or cyclic N-and / or C- terminally blocked peptides, which may make cleavage sites inaccessible due to steric hindrance (Eckart, 1994) . Moreover, the slight bacteriocin resistance to amylase and lipase might be an indication that no lipids or carbohydrate components are involved in the antibacterial activity (Torkar and Matijaši , 2003). The complete destruction of activity by the tested organic solvents, suggests that the bacteriocin molecule may not share similar hydrophobic properties with the other LAB bacteriocins (Klaenhammer, 1993). The unique properties of the produced bacteriocin stimulated further investigation of its molecular weight which was found to range from 3.496 to 6.512 KDa. This result was close with that obtained from the SDS-PAGE assay of some bacteriocins like megacin A-19213; being composed of two subunits, one of which is about 7.5 kDa in mass (Von Tersch et al., 1983), and to that reported for enterocin CRL35 (Wachsman et al., 2003).

Conclusion

Results obtained in this research may aid in understanding the industrial and technological significances of bacteriocins in reducing the risk of food contamination as the growth and proliferation of food borne pathogenic bacteria. Practical studies involving the type of medium/medium components that can affect the bacteriocin activity may be further required for exploring the value of the strain from the economical and technological point of view, for enhancing the microbial quality and safety of processed foods, and for developing new food products or processing systems.

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