Characterization of bacteriocin produced by *Lactobacillus rhamnosus* 1K isolated from traditionally fermented milk in the western highlands region of Cameroon.

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Abstract: *Lactobacillus rhamnosus* 1K, isolated from traditionally fermented milk from Cameroon, produces a bacteriocin active against food spoilage and pathogenic Gram-positive and Gram-negative bacteria including *Listeria innocua, Staphylococcus aureus, Salmonella typhi, Bacillus cereus, Streptococcus mutans, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia* and *Shigella flexneri*. Maximum bacteriocin activity (3200 AU/ml) was recorded in MRS broth after 8h at 30 °C with an initial pH of 5.5. The bacteriocin was resistant to treatments with α-amilase, lipase, lysozyme, surfactants, organic solvents, NaCl and EDTA. Furthermore, the bacteriocin remained active at 121° C for 30 min, 8 months of storage at -20 °C and under acidic, neutral, alkaline conditions. The mode of action against *Lactobacillus plantarum* 3SH is bacteriostatic. Increased bacteriocin production by *Lb. rhamnosus* 1K was noted at an initial pH of 6.0 or 6.5 and when MRS broth was supplemented with glucose (30 and 50 g/l) and tween 80 (1% and 4%). In addition, a lower bacteriocin production was noted in BHI broth and skim milk.

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1. INTRODUCTION

In the western highlands region of Cameroon, traditionally fermented milk is prepared by mean of natural fermentation by the Bororo cattle breeders. This product is nowadays most popular and widely consumed, not only as a beverage but also as an essential food. Traditional Fermented milk is made from freshly collected unpasteurized cow milk, allowed to ferment spontaneously for 1 – 2 days at room temperature. As revealed by our preliminary studies, lactic acid bacteria (LAB) are the predominant microorganisms (Zambou *et al.*, 2007).

Lactic acid bacteria (LAB) are known to produce a variety of antimicrobial compounds, of which bacteriocins are the most promising as they can be used as natural and safe food preservatives. Bacteriocins are ribosomally synthesized peptides that not only inhibit bacteria closely related to the producer strain but also food-borne pathogens and spoilage bacteria (Klaenhammer, 1988). Currently, due to consumer demand for reduction of chemical additives, bacteriocins have attracted increasing interest. Although several LAB strains have been reported as bacteriocin producers, their affectivity ranges from narrow to broad spectrum types. Also, some bacteriocins could exhibit their antibacterial activity not only under acidic conditions, but also under neutral and/or weak alkaline conditions (Hata *et al.*, 2009). Therefore, Biochemical characterization of bacteriocins in terms of their stability, host range and mode of antimicrobial action is essential so as to evaluate their possible potential as food preservatives. *Lactobacillus rhamnosus* strains are clinically important LAB widely used as probiotics; though being found in various habitats, only a few have been listed as producers of bacteriocins (Avonts *et al.*, 2004; Todorov and Dicks, 2005a) and still, little is known about the growth conditions required for their optimal bacteriocin production. Given that natural fermentation is difficult to control and sometimes produces unwanted products or products with short shelf life and contaminated by pathogens (Zambou, 2008), the use of bacteriocin producing LAB strains as starter or adjunct cultures during fermentation could be an alternative to overcome this major problem.

The objective of this study was the detection and partial characterization of a bacteriocin produced by *Lb. rhamnosus* 1K strain isolated from traditional fermented milk in order of its application as food preservatives. Furthermore, the optimal conditions for bacteriocin production were determined.

2. MATERIAL AND METHODS

2.1. Bacterial strains, media and growth conditions

Lactobacillus rhamnosus strain (given code 1K) used in this study was selected from a group of 35 lactic acid bacteria (LAB) strains previously isolated from traditional fermented milk produced in the western highlands region of Cameroon and so belonging to our laboratory collection. The selection was based on phenotypical studies such as antagonistic activity against other lactobacilli as well as pathogenic bacteria, and safety properties involving antibiotic susceptibility, negative haemolysis and gelatinase activities. This strain was identified based on physiological and biochemical characteristics using API system (API 50 CHL gallery, BioMérieux, Marcy l'Etoile France). Genotypic Identification was carried out by rep-PCR fingerprinting as described by Mohammed et al., (2009). It was maintained in MRS broth (Lab M, United Kingdom) plus glycerol (30% v/v) at -20°C and sub-cultured twice in MRS broth for activation prior to experimental use. *Lactobacillus plantarum* 3SH and several other LAB from our laboratory collection were used as indicator strains. Agar and soft agar media were prepared by adding respectively 1.5 and 0.75% (w/v) granulated agar (Merck, Darmstadt, Germany) to broth media. For bacteriocin production and partial purification, a modified MRS broth medium (mMRS) was prepared by mean of various ingredients (from Oxoid, UK; Merck, Darmstadt, Germany; and Sigma, St Louis, USA), without beef extract and by replacing ammonium citrate with ammonium sulfate (Dortu, 2008). After adding all medium components, the mixture was dissolved and autoclaved.

2.2. Bacteriocin activity assay

Antimicrobial activity on solid medium was performed using the triple-agar-layer method described by Todorov and Dicks (2005b) with the difference that buffered MRS medium (0.2 M potassium phosphate buffer, pH 7.0) was used and no antibiotic was added. Log phase culture of *Lactobacillus rhamnosus* 1K strain was spotted onto buffered MRS agar plate and then recovered with a second layer of the same medium. After incubation at 30 °C for 48 h in anaerobiosis, the plate was overlaid with a third layer of soft MRS agar seeded with 15 µl of an overnight culture of presumptive LAB indicator strains. The plate was incubated anaerobically at 30 °C for 24 h and zone of inhibition surrounding the spot was observed. For the detection of bacteriocin activity in liquid medium, agar well diffusion assay (AWDA) was used (Schillinger and Lücke, 1989). A 15-hour-old culture (2% v/v) of strain 1K was inoculated in buffered mMRS broth and incubated anaerobically at 30 °C for 15 h. The culture was centrifuged at 7,000 rpm for 30 min at 4 °C and supernatant collected. In order to destroy residual cells, prevent possible antimicrobial activity caused by hydrogen peroxide and degradation of bacteriocins by proteases, the supernatant was treated at 80 °C for 10 min (Todorov and Dicks, 2009a). Soft MRS agar seeded with the indicator strain (15 µl of 15-hour-old culture per 5 ml agar, i.e. approximately 10^7 CFU/ml) was dispensed onto pre-poured MRS agar plates. Six millimeter diameter wells were punched in the plates and filled with 50 µl of supernatant. After incubation of the plates anaerobically at 30 °C for 24 h, diameters of zone of inhibition were measured. Bacteriocin titer was determined by the agar- spot-test as described by Van Reenen *et al.* (1998) and expressed as arbitrary units (AU/ml). One AU was defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition of the indicator strain.

2.3. Bacteriocin production and protease activity

A 15-hour-old culture of *Lb. rhamnosus* 1K strain was inoculated (2% v/v) into buffered mMRS broth and incubated at 30 °C without agitation. Changes in cell density ($O.D_{600nm}$) were recorded every 2 h and bacteriocin activity was measured at the same time interval. For the protease activity assay, casein agar plate method (Schumacher and Schill, 1972) was used.

2.4. Partial Purification of the bacteriocin

Strain 1K was grown in 300 ml mMRS broth at 30 °C, until early stationary phase corresponding to maximum bacteriocin production. Cells were harvested by centrifugation (10,000 rpm, 15 min, 4 °C) and the bacteriocin precipitated from the cell-free supernatant with ammonium sulphate (Merck, Darmstadt, Germany) at 60% saturation. The mixture was stirred at 4 °C for at least 4 h, kept at 4 °C for overnight and proteins were harvested (10,000 rpm, 15 min, 4 °C). Precipitated proteins in the pellet and floating on the surface were collected and dissolved in 25mM ammonium acetate buffer (pH 6.5). All samples were stored at - 20 °C.

2.5. Sensitivity of bacteriocin to enzymes, heat, pH, storage, organic solvents, surfactants, NaCl, and EDTA.

In this set of experiment, ammonium sulphate precipitate was obtained from 100 ml cell-free supernatant, then dissolved in 50 ml of 25mM ammonium acetate buffer (pH 6.5) and used for assays. This partially purified (50 µl) bacteriocin was treated with trypsin (in 0.05 M Tris-HCl buffer, pH 8.0, Fluka Biochemika, Buchs, Switzerland), proteinase K (in 0.05 M phosphate buffer, pH 7.0, Merck, Darmstadt, Germany) α-Amylase (in 0.05 M phosphate buffer, pH

7.0, Sigma-Aldrich, Steinheim, Germany), lipase (in 0.05 M phosphate buffer, pH 7.0, Sigma-Aldrich, Steinheim, Germany) and lysozyme (in 0.05 M phosphate buffer, pH 7.0, Fluka Biochemika, Buchs, Switzerland) at 0.1 mg/ml and 1mg/ml final concentrations, and incubated at 37 °C for 2 h. Enzymes reactions were terminated by boiling for 5 min.

To test the pH stability, the partially purified bacteriocin was incubated at 37 °C for 2 h at pH 2.0 to 10.0 (at increments of one pH unit).

The effect of temperature on the bacteriocin was tested by heating the partially purified bacteriocin at 100 °C for 60 and 120 min, and at 121 °C for 15 and 30 min. In a separate experiment, the effect of surfactant on the bacteriocin activity was determined by adding surfactants (1% w/v final concentration) consisting of SDS (Sigma-Aldrich, Steinheim, Germany), Tween 80 (Merck, Darmstadt, Germany), Tween 20 (Merck, Darmstadt, Germany), Triton X-100 (Merck, Darmstadt, Germany) and Urea (Sigma-Aldrich, Steinheim, Germany) to the lyophilized partially purified bacteriocin. Ethylene diamine tetraacetic acid (EDTA, Boehringer Mannheim, Germany) was added to the sample at 0.1, 1, 2, 3, 4 and 5% final concentrations, while NaCl was added respectively at 1, 2, 3, 4, 5, 6 and 7% final concentrations. The prepared samples were incubated at 37 °C for 5 h. The effect of organic solvents was tested by adding 5% (v/v) solution of methanol, ethanol, isopropanol, acetone, chloroform and acetonitrile (all from Merck, Darmstadt, Germany) to the samples followed by incubation at 37 °C for 2 h. Finally, sample was kept at 4 °C to evaluate the effect of storage. Untreated sample and sterile mMRS broth treated with enzymes or chemicals served as controls; after each treatment, the residual activity was determined by AWDA. All experiments were performed in triplicate.

2.6. Mode of bacteriocin action

Strain 1K was anaerobically cultured in buffered mMRS broth for 10 h at 30 °C and the cell-free supernatant was obtained and treated as previously described. Twenty milliliter of this supernatant was added to 100 ml of a 3-hour growing culture of *Lactobacillus plantarum* 3SH in mMRS broth at 30 °C. Changes in cell density were recorded at 600 nm at 1-hour interval for 8 h.

2.7. Spectrum of inhibitory activity

The antibacterial activities of the cell-free supernatant and the ammonium sulfate-precipitated samples were tested against Gram-positive and Gram-negative bacteria. The indicator strains (0.5 Mac Farland suspensions) were inoculated in the appropriate soft agar media and the antibacterial activities were determined by AWDA previously described. All experiments were conducted in triplicate.

2.8. Optimization of bacteriocin production by Lb. rhamnosus 1K *strain*

2.8.1. Effect of inoculum size on bacteriocin production

To study the effect of inoculum size on bacteriocin production, a 15-hour-old culture of strain 1K was inoculated at different sizes (1%, 2%, 4%, 6%, 8% and 10% v/v) into buffered mMRS broth and incubated at 30°C for 10 h without agitation. The bacteriocin titer was expressed as arbitrary units (AU/ml) by the agar-spot test.

2.8.2. Optimum pH and temperature of bacteriocin production

The effect of initial medium pH was determined by inoculating the strain in buffered mMRS broth at various pH (4.5; 5.0; 5.5; 6.0; 6.5), followed by incubation at 30°C for 10 h. Separately, the optimum temperature of bacteriocin production was determined by inoculating strain 1K in buffered mMRS broth (pH 5.5) and incubation held respectively at 25°C, 30°C and 37°C for 10h, without agitation. Samples were taken every 2 h and examined for cell growth ($O.D_{600nm}$) as well as bacteriocin activity (AU/ml) using agar-spot-test as described above.

2.8.3. Effect of different growth media and medium composition on bacteriocin production

In another set of experiments, the effects of different growth media and medium composition were monitored. A 15-hour-old culture of *Lb. rhamnosus* 1K strain was used to inoculate (2% v/v) the following buffered (pH 5.5) growth media:

(a) mMRS broth: Tryptone (10.0 g/l), yeast extract (5.0 g/l), Glucose (20.0 g/l), K_2HPO_4 (2.0 g/l), sodium acetate (5.0 g/l), $MgSO_4$-$7H_2O$ (0.2 g/l), $MnSO_4$-H_2O (0.05 g/l), ammonium sulfate (2.0 g/l), Tween 80 (1ml/l); **(b)** mMRS broth (without tryptone and yeast extract), supplemented with each of the following: bacteriological peptone (20 g/l), tryptone (20 g/l), beef extract (20 g/l), yeast extract (20 g/l), tryptone (12.5 g/l) plus beef extract (7.5 g/l), tryptone (12.5 g/l) plus yeast extract (7.5 g/l), beef extract (10.0 g/l) plus yeast extract (10.0 g/l), and a combination of tryptone (10.0 g/l), beef extract (5.0 g/l) and yeast extract (5.0 g/l) respectively; **(c)** mMRS broth (without glucose), supplemented with glucose (20.0, 30.0 and 50.0 g/l), 20.0 g/l sucrose, 20.0 g/l lactose and 20.0 g/l maltose respectively; **(d)** mMRS broth (without Tween 80), supplemented with Tween 80 (2% and 5%); **(e)** mMRS broth without Sodium acetate, $MgSO_4$ and $MnSO_4$; **(f)** non buffered mMRS broth (pH

6.5), BHI (Brain Heart Infusion, Merck, Darmstadt, Germany) broth and skim milk (Leader Price, Gretz-Armainvilliers, France). All growth media were incubated at 30°C for 10h except skim milk, without agitation and aliquots were taken at the end of incubation period for cell growth recording and bacteriocin activity.

3. RESULTS AND DISCUSSION

3.1. Bacteriocin activity assay

Lb. rhamnosus 1K and its heat treated neutralized cell-free supernatant demonstrated antimicrobial activity against *Lactobacillus plantarum* 3SH and other indicator strains tested.

3.2. Bacteriocin production and protease activity.

Lb. rhamnosus 1K Strain showed growth stability at the stationary phase. Bacteriocin activity was detected at early logarithmic phase and reached its maximum (3200 AU/ml) at the stationary phase **(figure 1)**. Results of the protease activity showed that no extracellular proteases were produced (result not shown). Detection of bacteriocin activity at early logarithmic growth phase may suggest that the peptide is secreted as a primary metabolite. It has been reported that activity in broth cultures reached maximum only after the exponential growth had ceased (Jose *et al*., 1998), what is in tune with the present results. Similar observations were also reported for *Lb. rhamnosus* ST461BZ and ST462BZ (Todorov *et al.*, 2005a) and *Lb. rhamnosus* GP1 (Sarika *et al*., 2010).

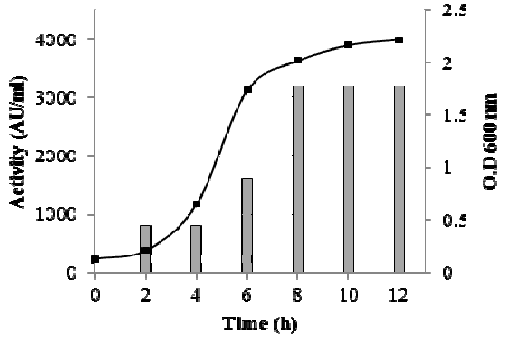

Figure 1: Growth of *Lactobacillus rhamnosus* 1K strain in buffered MRS broth at 30°C (-●-) and bacteriocin production (-■-).

3.3. Sensitivity of bacteriocin to enzymes, pH, heat, storage, organic solvents, surfactants NaCl, and EDTA.

The partially purified bacteriocin was inactivated after digestion with proteinase K and trypsin (**Table 1**). This suggested that the inhibitory compound is of proteinaceous nature and can thus be classified into the category of the bacteriocins. Treatment with α-Amylase and lipase did not affect the antimicrobial activity, suggesting that the bacteriocin is not attached to a carbohydrate or lipid moiety (**Table 1**). The partially purified bacteriocin retained full antibacterial activity in acidic, neutral and alkaline conditions (pH 2.0-10.0). The bacteriocin was resistant to heat. Remarkably, 80% of the activity could still be recorded against *Lb. plantarum* strain 3SH after 30 min at 121 °C. Full bacteriocin activity was retained upon storage at 4 °C up to 8 months (**Table 1**). Partially purified bacteriocin was not sensitive to NaCl, Tween 80, Tween 20 and Triton X-100. However, SDS reduced bacteriocin activity. When EDTA was added to the partially purified bacteriocin, the antimicrobial activity of the mixture was stronger than EDTA or bacteriocin tested alones (**Table 1**). This molecular stability of bacteriocins produced by *Lb. rhamnosus* 1K is quite similar to pediocin-like bacteriocins characteristics, thus suggesting that this bacteriocin might belong to class II bacteriocins described by Klaenhammer (1988).

3.4. Mode of bacteriocin action

The addition of crude bacteriocin to a 3-hour-old culture of *Lb. plantarum* 3SH strain resulted in growth inhibition and decrease in cell viability for 2 h, after which the strain recovered from the treatment (**Figure 2**). As reported by Thonart *et al.* (2009) many indicator strains (e.g. *L. monocytogenes*) have been also found to bounce some hours after bacteriocins treatments. From this result, we can conclude that bacteriocin produced by *Lb. rhamnosus* 1K has a bacteriostatic mode of action against *Lb. plantarum* 3SH.

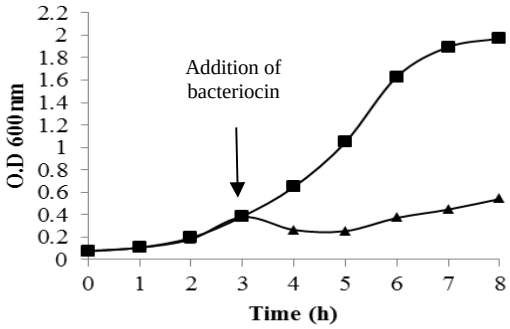

Figure 2: Effect of bacteriocin on the growth of *Lb. plantarum* 3SH; Optical Density (O.D) in the absence (-■-) and presence (-▲-) of bacteriocin.

3.5. Spectrum of inhibitory activity

The Neutralized cell-free supernatant and partially purified bacteriocin inhibited the growth of a *Lb. plantarum* strain as well as a number of food spoilage and foodborne pathogenic bacteria (**Table 2**). In most cases, the partially purified bacteriocin showed

significantly higher activity (P<0.05) as compared to the supernatant, due to the high concentration of bacteriocin in the ammonium sulfate precipitate.

Table 1: Effect of enzymes, pH, heat, storage, organic solvents, surfactants, Urea, NaCl and EDTA on the antibacterial activity of the partially purified bacteriocin.

Treatment		Inhibition Zone (mm)[a]
Control		14.0 ± 0.0
Enzymes		
	Trypsin (0.1 & 1 mg/ml)	-
	Proteinase K (0.1 & 1 mg/ml)	-
	α-Amylase, Lipase, Lysozyme (0.1 & 1 mg/ml)	14.0 ± 0.0
pH		
	2 - 10	14.0 ± 0.0
Temperature		
	100 °C for 60 min	13.5 ± 0.7
	100 °C for 120 min	11.0 ± 0.0
	121 °C for 15 min	11.0 ± 1.4
	121 °C for 30 min	10.0 ± 1.4[a]
Storage		
	4 °C for at least 8 months	14.0 ± 0.0
Organic solvents		
	Ethanol, Methanol, Acetone, Isopropanol	14.0 ± 0.0
	Acetonitrile, Chloroform	13.5 ± 0.7
Surfactants / Chemicals		
	SDS (1% w/v)	10.0 ± 0.0[b]
	Tween 80, Tween 20, Triton X-100, Urea (1% w/v)	14.0 ± 0.0
	NaCl (1 – 7% w/v)	14.0 ± 0.0
	EDTA (0.1% w/v)	2.0 ± 0.0[c]
	EDTA (2% w/v)	1.0 ± 0.0 [c]
	EDTA (5% w/v)	2.0 ± 0.0 [c]

[a]: Inhibition Zone Diameters, including the diameter of the wells (6 mm). ''–": no inhibitory zone was observed.
[a,b,c]: The values carrying this symbol differ significantly (p<0.05) from the control.

Bacteriocins are proteins which are often bactericidal to Gram-positive bacteria (Todorov and Dicks, 2005b), usually closely related bacteria. However in this study, many Gram- negative bacteria where inhibited as also reported by other studies (Todorov *et al.*, 2007; Luo *et al.*, 2011). More interestingly, Multi-Drug Resistant (MDR) strains of *Staphylococcus aureus* and *Escherichia coli* were also inhibited. No activity was detected against many other *Lactobacilli* as well as an *Enterococcus faecium* strain tested (**Table 2**), suggesting that *Lb. rhamnosus* 1K strain could be easily associated with other starter cultures.

Table 2: Spectrum of antibacterial activity of the bacteriocin produced by *Lb. rhamnosus* 1K strain.

Indicator strains	Source	Growth conditions	Bacteriocin Activity (mm)*	
			NCFS	ASP
lactic acid bacteria				
Lb. Plantarum 3SH	Our isolate	MRS, 30°C	14.0 ± 0.0[b]	18.0 ± 0.0[b]
Lb plantarum 9S	Our isolate	MRS, 30°C	-	-
Lb. plantarum 29V	Our collection	MRS, 30°C	-	-
Lb. rhamnosus 18S	Our isolate	MRS, 30°C	-	-
Lb. fermentum 2K	Our isolate	MRS, 30°C	-	-
Enterococcus faecium	DSM 13596	BHI, 37°C	-	-
Gram positive pathogenic bacteria				
Listeria innocua	ATCC 33090	BHI, 37°C	12.0 ± 0.0[b]	14.5 ± 0.7[b]
Staphylococcus aureus	ATCC 25923	BHI, 37°C	13.0 ± 1.4	15.0 ± 0.0
Staphylococcus aureus	ATCC 25922	BHI, 37°C	16.0 ± 0.0[c]	19.0 ± 0.0[c]
Staphylococcus aureus (MDR)	Clinical isolate	BHI, 37°C	11.5 ±0.7[b]	16.0 ± 0.0[b]
Bacillus cereus	ATCC 11778	BHI, 37°C	12.0 ± 0.0[c]	13.0 ± 0.0[c]
Streptococcus mutans	DSM 20523	BHI, 37°C	16.0 ± 0.0[c]	20.0 ± 0.0[c]
Gram negative pathogenic bacteria				
Escherichia coli	ATCC 13706	BHI, 37°C	12.0 ± 0.0[a]	15.0 ± 1.4[a]
Escherichia coli (MDR)	Clinical isolate	BHI, 37°C	12.0 ± 0.0[a]	13.5 ±0.7[a]
Salmonella typhi	ATCC 6539	NB, 37°C	13.0 ± 0.0[c]	14.0 ± 0.0[c]
Pseudomonas aeruginosa	ATCC 20027	BHI, 37°C	14.0 ± 0.0[a]	15.5 ± 0.7[a]
Pseudomonas aeruginosa	ATCC 27853	BHI, 37°C	11.0 ± 1.4	13.0 ± 0.0
Klebsiella pneumoniae	Clinical isolate	BHI, 37°C	11.5 ± 0.7[a]	13.0 ± 0.0[a]
Shigella flexneri	Clinical isolate	NB, 37°C	12.5 ± 2.1	13.5 ± 0.7

*: Inhibition zone Diameters are means of triplicates; Wells (6 mm in diameter) were filled with 100 µl of supernatant or 60% ammonium sulfate precipitated samples. "-" no inhibition. **MDR** = Multi-Drug Resistant. **NCFS** = Neutralized Cell-free supernatant; **ASP** = Ammonium Sulfate Precipitate.
DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.
ATCC: American Type Culture Collection, Manassas, VA, USA.
MRS: de Man, Rogosa and Sharpe broth.
BHI: Brain Heart Infusion broth.
NB: Nutrient Broth
[a]: the values of these lines differ significantly (P<0.05)
[b]: the values of these lines differ significantly (P<0.01)
[c]: the values of these lines differ significantly (P<0.001)

3.6. Optimization of bacteriocin production

3.6.1. Effect of inoculum size on bacteriocin production

When MRS broth medium was inoculated with *Lb. rhamnosus* 1K at different sizes, the same activity level (3200 AU/ml) was recorded (data not shown).

3.6.2. Optimum pH and temperature of bacteriocin production

Irrespective of the temperature or pH, bacteriocin production increases with time during the 8 h of growth. The maximum bacteriocin activity was recorded in buffered mMRS broth (3200 AU/ml after 8 h) at 30 °C, compared to 1600 and 800 AU/ml obtained at 25 °C and 37 °C respectively (**Fig 3**), even though the strain grew very well at these temperatures (results not shown). Similar results were also reported by Sarika *et al.* (2010), thus confirming the suggestion that growth temperature plays an important role in bacteriocin production. Bacteriocin production was also affected by the initial pH of the medium. Increased in bacteriocin production (12800 AU/ml after 8 h) was recorded when the initial pH of the medium was 6.0 or 6.5, whereas the same level of production (3200 AU/ml after 8 h) was observed at pH 4.5, 5.0 and 5.5 (**Fig 3**). These results showed that bacteriocin production by *Lb.rhamnosus* 1K is stimulated at an initial pH of 6.0 or 6.5. Similar results were obtained with other bacteriocins from *Lb. rhamnosus* or *Lb. plantarum* strains (Todorov and Dicks, 2005a; von Mollendorf *et al.*, 2006; Todorov *et al.*, 2011). The use of bacteriological peptone, tryptone, beef extract and yeast extract as sole nitrogen source resulted in a bacteriocin production of 3200, 1600, 1600 and 400 AU/ml respectively. The combination of tryptone and beef extract, tryptone and yeast extract yielded 3200 AU/ml, whereas the combination of beef extract and yeast extract, tryptone, beef extract and yeast extract yielded 1600 AU/ml (**Table 3**). So far, we can conclude that bacteriological peptone is the best organic nitrogen source for this bacteriocin production. Similar results have been reported for other bacteriocins, in which case optimal production was obtained in MRS broth supplemented with bacteriological peptone, followed by casamino acids, tryptone and meat extract (Pingitore *et al.*, 2009). Growth of *Lb. rhamnosus* 1K in the presence of 20 g/l of glucose and sucrose yielded 1600 AU/ml, whereas in the presence of lactose and maltose 3200 AU/ml was recorded. However, supplementation of the medium with glucose (30 g/l and 50 g/l) resulted in an increase in bacteriocin production (12800 AU/ml). Increase in bacteriocin production due to glucose supplementation have been also reported for other bacteriocins from *Lb. plantarum* (Todorov *et al.*, 2011).

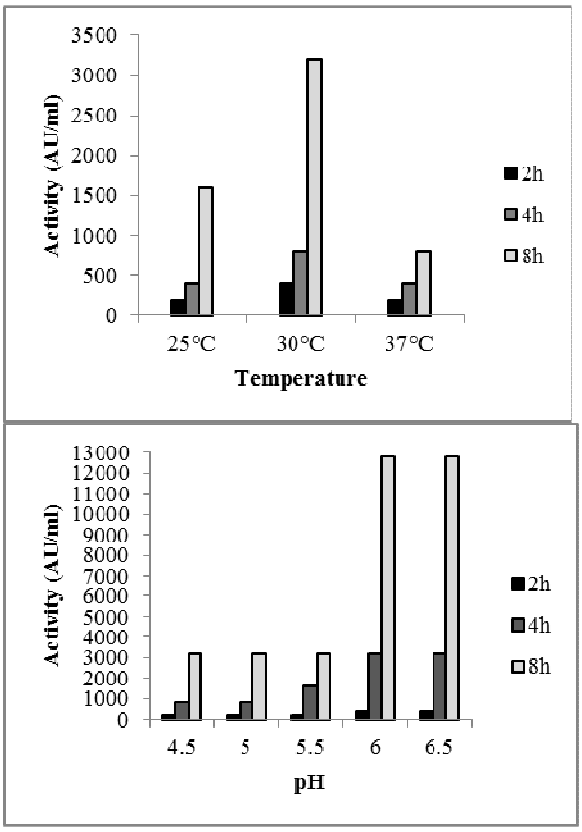

Figure 3: Effect of pH and temperature on the bacteriocin production by *Lb. rhamnosus* 1K at different time intervals.

The absence of Tween 80 in mMRS broth lead to reduction in bacteriocin production (800 AU/ml), whereas supplementation of the medium with Tween 80 (2.0 and 5.0 ml/l) resulted in an increase in bacteriocin production (12800 AU/ml) (**Table 3**). Being a surfactant, Tween 80 may prevent the adsorption of the bacteriocin to the producer cell. Simultaneous exclusion of CH_3COONa, $MgSO_4$ and $MnSO_4$ from the medium resulted in a significant decrease in bacteriocin production, since 200 AU/ml was recorded (**Table 3**). This drastic effect might be due to the absence of $MnSO_4$ particularly; Exclusion of $MnSO_4$ from MRS medium also had a negative effect on both the production of bacJW6BZ and bacJW11BZ (von Mollendorf *et al.*, 2009), bacteriocin ST202Ch and bacteriocin ST216Ch (Todorov *et al.*, 2010). In fact, in the medium, $MnSO_4$ provides cations used in metabolism. The same level of bacteriocin activity (3200 AU/ml) was recorded when strain 1K was grown in buffered and non buffered mMRS broth medium whereas a low levels were detected in BHI and skim milk, as 200 and 100 AU/ml respectively were recorded.

Table 3: Influence of medium components and different growth media on the growth bacteriocin production by *Lb. rhamnosus* 1K at 8 h, 30 °C against *Lb. plantarum* 3SH.

	$O.D_{600\ nm}$	Activity (AU/ml)
Organic nitrogen Sources		
Bacteriological peptone (20 g/l)	1.090 ± 0.176	3200
Tryptone (20 g/l)	1.606 ± 0.020	1600
Beef extract (20 g/l)	1.703 ± 0.025	1600
Yeast extract (20 g/l)	1.813 ± 0.002	400
Tryptone + Beef extract (12.5 + 7.5 g/l)	1.704 ± 0.065	3200
Tryptone + Yeast extract (12.5 + 7.5 g/l)	1.625 ± 0.020	3200
Beef extract + Yeast extract (10.0 + 10.0 g/l)	1.745 ± 0.077	1600
Tryptone + Beef extract + Yeast extract (10.0 + 10.0 + 5.0 g/l)	1.725 ± 0.100	1600
Carbohydrates sources		
Glucose (20 g/l)	1.743 ± 0.025	1600
Sucrose (20 g/l)	1.700 ± 0.100	1600
Lactose (20 g/l)	1.551 ± 0.033	3200
Maltose (20 g/l)	1.458 ± 0.061	3200
Glucose (30 g/l)	1.514 ± 0.074	12 800
Glucose (50 g/l)	1.194 ± 0.082	12 800
Tween 80 and salts		
Tween 80 free mMRS	1.548 ± 0.064	800
Tween 80 (1.0 ml/l)	1.625 ± 0.020	3200
Tween 80 (2.0 ml/l)	1.172 ± 0.083	12 800
Tween 80 (5.0 ml/l)	1.367 ± 0.050	12 800
CH_3COONa, $MgSO_4$ and $MnSO_4$ free mMRS	1.329 ± 0.049	200
Different growth media		
mMRS (buffered)	1.625 ± 0.020	3200
mMRS (non buffered, pH adjusted to 6.0)	1.690 ± 0.001	3200
BHI (buffered)	0.995 ± 0.043	200
Skim milk (125 g/l)	ND	100

ND: Not Determined

Similar results were also recorded for bacteriocins ST23LD and ST341LD (Todorov and Dicks, 2006d), bacJW6BZ and bacJW11BZ (von Mollendorf *et al.*, 2009). This further confirms the claim that specific nutrients are required for the production of bacteriocins.

4. Conclusion

The bacteriocin produced by *Lb. rhamnosus* 1K strain showed high stability to various factors as well as a broad antibacterial spectrum. Although at lower level, Strain 1K produces bacteriocin in skim milk and increased bacteriocin production was recorded when MRS broth medium was supplemented by glucose or Tween 80. Our results highly suggest that strain 1K or its bacteriocin can be applied to the process of fermented dairy or non-dairy products to inhibit the growth of spoilage and pathogenic bacteria during fermentattion. Further research is necessary for study the application of this strain.

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