Characterization of a Bacteriocin Produced by *Lactobacillus plantarum* Lp6SH Isolated from "*Sha'a*", a Maize-Based Traditionally Fermented Beverage from Cameroon

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Abstract

Lactobacillus plantarum Lp6SH isolated from "*Sha'a*" a maize-based traditionally fermented beverage from Cameroon, produces a bacteriocin active against 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*. Highest bacteriocin production in buffered MRS broth medium was achieved after 6 h of incubation without agitation. The bacteriocin is of proteinaceous nature as judged by the complete loss of activity after treatment by proteolytic enzymes. Interestingly, this bacteriocin was stable at 121°C for 30 min, over a wide range of pH (2.0-10.0), and when treated with surfactants, organic solvents, EDTA and NaCl. The activity remained after 8 months of storage at 4°C and the mode of action against *Salmonella typhi* ATCC 6539 was bactericidal. The bacteriocin was partially

purified using ammonium sulphate precipitation, gel filtration and cation exchange chromatographies. Mass Spectrometry Analysis showed that the Molecular weight of the bacteriocin is 2.340 kDa.

Keywords: *Sha'a*, *Lactobacillus plantarum*, Bacteriocin, Characterization

1. Introduction

Fermentation is one of the oldest and most economical methods of producing and preserving foods (Billings, 1998; Chavan and Kadam, 1989). In developing countries, many traditional foods and beverages are prepared by mean of natural or spontaneous fermentation. In Cameroon, some examples are fermented milk called "*Kossam*" and a maize-based fermented beverage called "*Sha'a*", which is most popular and widely consumed in the western highlands of the country. "*Sha'a*" is a sweet or low-alcoholic beverage which is viscous, effervescent and whitish-grey to brown coloured. Due to it thick consistency, it is also considered as food by the consumers. As revealed by our preliminary studies, the dominant microorganisms in these beverages are lactic acid bacteria (LAB) and yeasts. During fermentation, many of the LAB produce antimicrobial substances, including bacteriocins. As defined by Klaenhammer (1988), bacteriocins produced by LAB are ribosomally synthesized extracellular small peptides that exhibit bactericidal and bacteriostatic activity against genetically closely related bacteria. Bacteriocins received particular interest due to their potential application in the food industry as natural preservatives, given that they have the advantage of being reliable, non toxic to eukaryotic cells and rapidly digested by proteases within the gastrointestinal tract (Parada *et al.,* 2007). Bacteriocins have always been said to be active only against gram-positive bacteria. However, different researchers described bacteriocins also active against gram-negative bacteria (Ivanova *et al.,* 2000; Kabadjova *et al.,* 2000; Gong *et al.,* 2010). Microbial contamination is still a major problem in local and modern food industries, and more than 250 food-borne diseases have been described. Also, nowadays, there is a worldwide growing demand by consumers for foods containing less or no chemical preservatives. Our preliminary study on the assessment of microbiological quality of "*Sha'a*" showed that many samples were found contaminated with undesirable microorganisms, whereas other were safe; This stimulated further investigation leading to isolation of LAB with antagonistic activity (Zambou *et al.,* 2007). Despite this, no published paper to the best of our knowledge addressed selection of starter culture for production of "*Sha'a*" as well as study on bacteriocin production by LAB from this beverage. Whereas there is obvious evidence that LAB strains from different origins could possess antimicrobial activities at different levels. This further stimulates great interest and research on naturally produced antibiotics such as novel bacteriocins from LAB for their various applications.

This study reports for the first time the partial purification and physico-chemical characterization of a bacteriocin produced by a LAB strain (*Lb. plantarum* Lp6SH) isolated from "*Sha'a*".

2. Material and Methods

2.1 Bacterial strains, media and growth conditions

The LAB consisted of 21 *Lactobacillus spp* strains isolated from fermented milk and "*Sha'a*", previously selected based on their antagonistic activity against each other and some pathogenic indicator bacteria, safety properties and identified as *Lactobacillus plantarum* (72%), *Lactobacillus rhamnosus* (8%), *Lactobacillus fermentum* (67%) and *Lactobacillus coprophilus* (33%) based on phenotypic characteristics and rep-PCR fingerprinting. The indicator strain was screened among the 21 mentioned above. These strains were kept in MRS broth plus glycerol (70:30) at -20 °C and were sub-cultured twice in MRS broth (Lab M, United Kingdom) for activation prior to experimental use. Agar and soft agar media were prepared by adding respectively 1.5 and 0.75% (w/v) granulated agar (Merck) to broth media. For bacteriocin purification, a modified MRS broth medium (mMRS) was prepared by mean of various ingredients (from Oxoid, Merck and Sigma), without beef extract and by replacing ammonium citrate with ammonium sulfate. After adding all medium components, the mixture was dissolved and autoclaved.

2.2 Screening for bacteriocin producing strains

This was performed using the triple-agar layer method described by Todorov and Dicks (2005) 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* strains were spotted on buffered MRS agar plates and then recovered with a second layer of the same medium. After incubation at 30°C for 48 h in anaerobiosis, the plates were overlaid with a third layer of soft MRS agar seeded with 15 µl of an overnight culture of presumptive LAB indicator strains. The plates were incubated anaerobically at 30°C for 24 h and zone of inhibition surrounding the spots were observed. The most sensitive strain, *Lb. plantarum* 3SH was selected as indicator for the next experiments.

2.3 Bacteriocin activity assay

Cell free supernatants of the selected producer strains were screened for bacteriocin activity by the agar well diffusion assay (AWDA) as described by Schillinger and Lücke (1989). A 15-hour-old culture (2% v/v) of each *Lactobacillus* strain was inoculated in buffered MRS broth and incubated anaerobically at 30°C for 10 h. The culture was centrifuged at 7000 rpm for 30 min at 4 °C and supernatants were collected, then treated at 80 °C for 10 min (Todorov and Dicks, 2009). Soft MRS agar seeded with the indicator strain (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. At the end of this set of experiments, *Lb. plantarum* Lp6SH strain showing the highest activity was selected for the following experiments.

2.4 Bacteriocin production and protease activity

A 15-hour-old culture of *Lb. plantarum* Lp6SH strain was inoculated (2% v/v) into buffered MRS 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.

2.5 Sensitivity of bacteriocin to enzymes, pH, temperature, 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 25 mM ammonium acetate buffer (pH 6.5) and used for assays. This partially purified bacteriocin was treated with proteolytic enzymes at the ratios 1:50 and 1:100 (Enzyme:Protein, µg/µg), followed by incubation at 37°C for 24 h on the one hand. On the other hand, 50 µl of partially purified bacteriocin was treated with trypsin (Fluka Biochemika), α-chymotrypsin (Sigma), Pepsin (Sigma), Proteinase K (Merck), α-Amylase (Sigma) and lipase (Sigma) 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, Tween 80, Tween 20, Triton X-100 and Urea to the lyophilized partially purified bacteriocin. Ethylenediaminetetraacetic acid (EDTA) 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 to the samples followed by incubation at 37°C for 2 h. Finally, the sample was kept at 4 °C to evaluate the effect of storage. Untreated sample and sterile MRS 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 Lp6SH was anaerobically cultured in buffered MRS broth for 6 h at 30°C and the culture free supernatant was obtained and treated as previously described. Twenty milliliter of this supernatant (800 AU/ml) was added to 100 ml of a 3-hour growing culture of *Salmonella enterica* subsp. *enterica* serovare Typhi (or *Salmonella* Typhi) strain ATCC 6539 in nutrient broth at 37°C Changes in cell density were recorded at 600 nm at 1-hour interval for 8 h, and the number of viable cells (CFU) was determined by plating the samples on *Salmonella-Shigella* agar (1.5% w/v) followed by incubation at 37°C for 24 h.

2.7 Partial Purification of the bacteriocin

The bacteriocin was purified from 1 litre of mMRS broth culture of *Lb. plantarum* Lp6SH strain incubated at 30 °C for 6 h, without agitation. The culture was centrifuged (7000 rpm, 30 min, 4°C) and the cell-free neutralized supernatant obtained was treated at 80°C for 10 min, and then precipitated with ammonium sulfate (60% saturation). The mixture was stirred at 4°C for at least 4 h and then kept at 4°C for overnight. The precipitate was collected by centrifugation (10 000 rpm, 15 min, 4°C), dissolved in one tenth volume of 25 mM ammonium acetate buffer (pH 6.5) and loaded (10 ml) on a sephadex G-25 (Pharmacia, Uppsala, Sweden)

column (30 cm x 2.5 cm) equilibrated with 0.1M acetic acid solution at a flow rate of 16ml/h. Fractions of 4 ml were collected and the antimicrobial activity was determined by the AWDA. Active fractions were pooled and loaded on a CM-cellulose (microgranular form, Sigma-Aldrich) column (30 cm x 1.5 cm) pre-equilibrated and washed with sterile deionised water. The absorbed proteins were eluted with a stepwise gradient of (0 – 1M) NaCl at a flow rate of 16 ml/h. Fractions of 4ml were collected, the absorbance recorded at 280 nm and the antimicrobial activity was determined.

2.8 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The pooled active fractions from gel filtration chromatography was analyzed by Tris-glycine SDS-PAGE (Laemmli, 1970) under reducing conditions and on a Bio-Rad Electrophoresis system, using 15% separation gel and 4% stacking gel (30% acrylamide, 0.8% bisacrylamide). Bovine Serum Albumine (66 kDa) and Lysozyme (14.3 kDa) were used as molecular mass markers. Electrophoresis was run at a constant voltage (150 V). The gel was cut into 2 halves; one half was visualized by Coomassie Brilliant Blue R-250 and silver staining. The other half was placed in a MRS agar plate and overlaid with *Lactobacillus plantarum* 3SH strain seeded in soft MRS agar, in order to determine the authenticity of the active bacteriocin.

2.9 Mass Spectrometry Analysis

Pooled Active peptide fraction from CM-Cellulose Chromatography was analysed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/ MS) (Ultraflex III *TOF/TOF*, Bruker, Daltonics, Bremen, Germany). A solution of 10 mg/ml of α-cyano-4-hydroxycinnamic acid was used as saturated matrix. The sample was diluted in 50% Acetonitrile/0,1%TFA solution and then, 0.5 µl of the diluted sample was mixed with an equal volume of saturated matrix solution. The mixture was spotted on the target of a 16×24 MALDI plate and left to dry at room temperature for at least 15 min. The analyzer operated in positive ion mode, Desorption/Ionization was realized by laser irradiation using nitrogen laser (λ = 337 nm) and the spectrum was acquired in reflectron mode.

2.10 Spectrum of inhibitory activity

The antibacterial activities of 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.

3 Results and Discussion

3.1 Screening for bacteriocin producing strains

Using combination of Triple-Agar layer method and AWDA, 12 (57.1%) out of the 21 strains tested were found to be bacteriocin producers. The strain *Lb. plantarum* 6S, whose supernatant exhibited highest and broad activity, was selected for next experiments. It was designated as *Lactobacillus plantarum* Lp6SH and the bacteriocin produced was termed bacteriocin Lp6SH. Likewise, strain *Lactobacillus plantarum* 3S, selected as most sensitive was designated as *Lactobacillus plantarum* 3SH.

3.2 Bacteriocin production and protease activity

The strain Lp6SH showed a typical sigmoidal growth response. Maximum activity was recorded at early stationary phase and decreases afterwards (figure 1). Results of the protease activity showed that extracellular proteases are produced during the stationary phase (data not shown). Thus, the decrease of bacteriocin production from the stationary phase can be ascribed to destruction by the proteases produced. Detection of bacteriocin production after 2 h of growth followed by maximum production at early stationary growth phase may suggest that the peptide is secreted as a primary metabolite. This is in accordance with results obtained for other bacteriocins from *Lb. plantarum* so far described (Van Reenen *et al.,* 1998; Todorov and Dicks, 2004; Todorov *et al.,* 2004; Todorov *et al.,* 2011).

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

Complete inactivation was observed when partially purified bacteriocin Lp6SH was treated by trypsin, α-chymotrypsin and pepsin, thus confirming its proteinaceous nature. 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). Similar results have been reported for other bacteriocins of *Lb. plantarum* (De Vuyst and Vandamme, 1994; Todorov *et al.,* 2004). Bacteriocin Lp6SH was found to be stable in the pH range 2.0 to 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 and Urea 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). Many other bacteriocins produced by *Lb. plantarum* strains were found to be stable to the above factors. However, plantaricin C19 produced by *Lb. plantarum* C19 lost its activity after treatment with SDS or Triton X-100 (Atrih, *et al.,* 2001).

3.4 Mode of bacteriocin Lp6SH action

The addition of crude bacteriocin to a 3-hour-old culture of *Salmonella* Typhi ATCC 6539 strain caused a significant decrease in cellular viability (from 3.8 x 10^6 CFU/ml to 1.9 x 10^4 CFU/ml within 5 h) during the 8 h, while the viability of untreated cells significantly increases in comparison (Figure 2). Killing of the cells started within 1 h after the addition. These results suggest that the mode of activity of bacteriocin Lp6SH is bactericidal.

3.5 Partial Purification of the bacteriocin

During the purification protocol, the proteins precipitated by ammonium sulfate presented activity (409 600 AU/ml) against *Lb. plantarum* 3SH. When the ammonium sulfate precipitate was subjected to gel filtration on a sephadex G-25 column, 12 800 AU/ml activity was recorded. During further purification on CM-Cellulose column, 0.6M NaCl removed the absorbed bacteriocin from the column and 400 AU/ml was obtained. A protocol similar to this one but without gel filtration was used for purification of diploccocin and other protocols have been also used by several authors for purification of bacteriocins produced by other *Lb. plantarum* strains (Todorov *et al.,* 1999; Gong *et al.,* 2010).

3.6 SDS-PAGE and mass spectrometry

No protein bands were detected by SDS- PAGE analysis. However, bacteriocin activity was detected in the gel after the overlay assay (Figure 3). This result suggests that the concentration of the active peptide was low as also found for *Lb. acidophilus* n.v. Er 317/402 strain narine (Mkrtchyan *et al.,* 2010). MALDI-TOF/MS analysis revealed that the molecular weight of the bacteriocin is 2.340 kDa.

3.7 Spectrum of inhibitory activity

Bacteriocin Lp6SH inhibited the growth of a *Lb. plantarum* strain as well as a number of food spoilage and foodborne pathogenic bacteria including species of the genera *Listeria*, *Streptococcus*, *Shigella, Bacillus*, *Staphylococcus, Escherichia, Salmonella, Pseudomonas* and *Klebsiella*. However, No activity was detected against many other *Lactobacilli* as well as an *Enterococcus faecium* strains tested (Table 2), suggesting that *Lb. plantarum* Lp6SH strain could be easily associated with other LAB starter cultures. More interestingly, Multi-Drug Resistant (MDR) strains of *Staphylococcus aureus* and *Escherichia coli* were inhibited. Bacteriocin Lp6SH is hence being part of the few ones produced by *Lb. plantarum* strains that inhibited the growth of Gram-negative bacteria (Todorov *et al.,* 2007; Gong *et al.,* 2010). But it could be particular in that it is active against *E. coli*, *P. aeruginosa*, *S.* Typhi and *K. pneumoniae* (Table 2). Inhibition of *Listeria spp* by bacteriocin Lp6SH suggests that it might belong to class IIa bacteriocins.

4. Conclusion

The bacteriocin produced by *Lb. plantarum* Lp6SH strain was partially purified. It showed high stability to pH, heat, surfactants, organic solvents and NaCl. In addition, Bacteriocin Lp6SH showed a broad antibacterial spectrum. *Lb. plantarum* Lp6SH may be used as protective stater culture to enhance the safety of spontaneously fermented foods.

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Table 1. Effect of enzymes, pH, heat, storage, organic solvents, surfactants, Urea, NaCl and EDTA on Bacteriocin Lp6SH activity

Treatment		**Inhibition Zone (mm)[a]**
Enzymes		
	Without Enzymes	15.0
	Trypsin, Pepsin , α-Chymotrypsin (1:50 & 1:100 µg/µg protein)	-
	Trypsin, Pepsin, α-Chymotrypsin, Proteinase K (0.1 & 1 mg/ml)	-
	α-Amylase, Lipase (0.1 & 1 mg/ml)	14.5
	Lysozyme (0.1 & 1 mg/ml)	15.0
pH		
	2 - 8	15.0
	10	14.5
Temperature		
	100 °C for 60 min	15.0
	100 °C for 120 min	14.0
	121 °C for 15 min	14.0
	121 °C for 30 min	12.0
Storage		
	4 °C for at least 8 months	15.0
Organic solvents		
	Ethanol, Methanol, Acetone	15.0
	Isopropanol, Acetonitrile, Chloroform	14.5
Surfactants / Chemicals		
	SDS (1% w/v)	9.0
	Tween 80, Tween 20, Triton X-100 (1% w/v)	15.0
	Urea (1% w/v)	14.5
	NaCl (1 – 7% w/v)	15.0
	EDTA (0.1% w/v)Without bacteriocin	12.0
	EDTA (0.1% w/v)With bacteriocin	16.0
	EDTA (2% w/v)Without bacteriocin	23.0
	EDTA (2% w/v)With bacteriocin	26.0
	EDTA (5% w/v)Without bacteriocin	27.0
	EDTA (5% w/v)With bacteriocin	31.0

[a]: Mean Inhibition Zone Diameters, including the diameter of the wells (6 mm). ‘‘–”: no inhibitory zone was observed.

Table 2. Spectrum of antibacterial activity of bacteriocin Lp6SH

Indicator strains	Source	Growth conditions	Bacteriocin Activity (mm)	
			NCFS	ASP
lactic acid bacteria				
Lb. Plantarum 3SH	Our isolate	MRS[c], 30°C	16	21
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. Rhamnosus 1K	Our isolate	MRS, 30°C	-	-
Lb. Fermentum 2K	Our isolate	MRS, 30°C	-	-
Enterococcus faecium	DSM[a] 13596	BHI[d], 37°C	-	-
Gram positive pathogenic bacteria				
Listeria innocua	ATCC[b] 33090	BHI, 37°C	15	19
Staphylococcus aureus	ATCC 25923	BHI, 37°C	17	22
Staphylococcus aureus (MDR)	Clinical isolate	BHI, 37°C	11	17
Bacillus cereus	ATCC 11778	BHI, 37°C	13	18
Streptococcus mutans	DSM 20523	BHI, 37°C	15	22
Gram negative pathogenic bacteria				
Escherichia coli	ATCC 13706	BHI, 37°C	13	18
Escherichia coli (MDR)	Clinical isolate	BHI, 37°C	-	8
Salmonella Typhi	ATCC 6539	NB[e], 37°C	16	24
Pseudomonas aeruginosa	ATCC 9027	BHI, 37°C	15	20
Pseudomonas aeruginosa	ATCC 27853	BHI, 37°C	13	17
Klebsiella pneumoniae	Clinical isolate	BHI, 37°C	15	18
Shigella flexneri	Clinical isolate	NB, 37°C	12	18

*: 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.

a: DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany

b: ATCC: American Type Culture Collection, Manassas, VA, USA

c: de Man, Rogosa and Sharpe

d: Brain Heart Infusion

e: Nutrient Broth

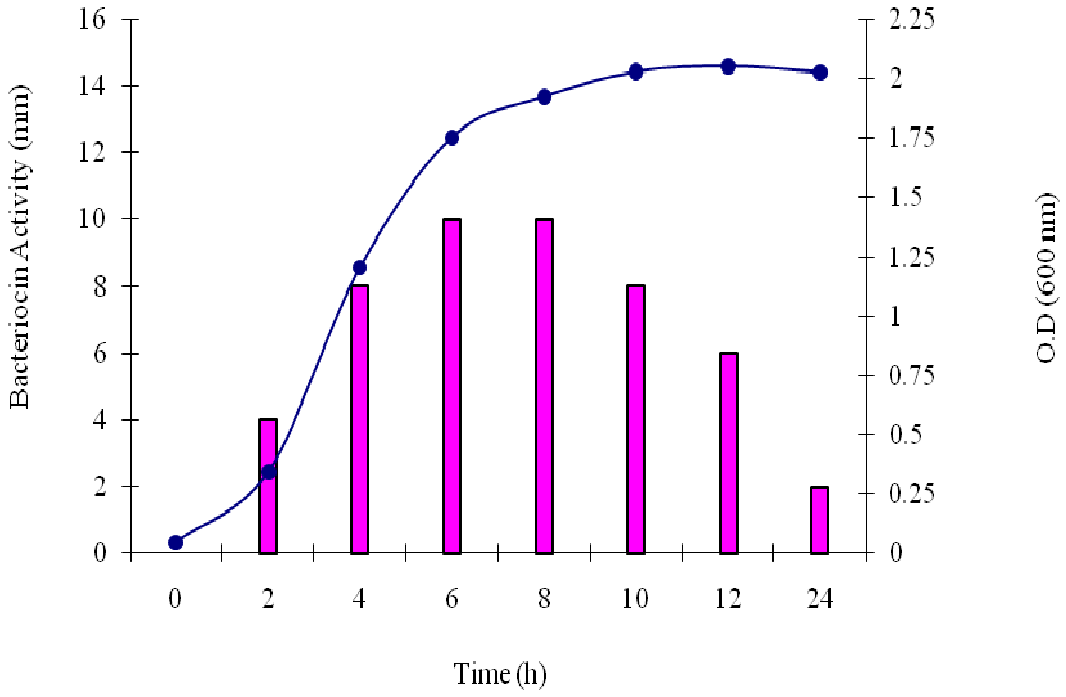

Figure 1. Growth of *Lactobacillus plantarum* Lp6SH strain in buffered MRS broth at 30°C (-●-) and bacteriocin production (-■-)

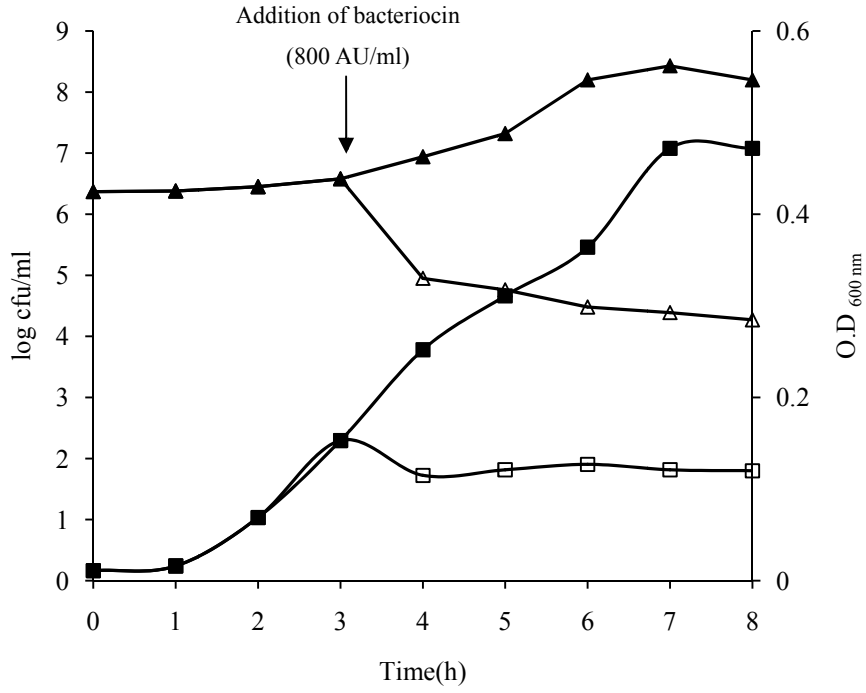

Figure 2. Effect of bacteriocin Lp6SH on the growth of *salmonella* Typhi ATCC 6539; Optical Density (O.D) in the absence (-■-) and presence (-□-) of bacteriocin; Viable cell counts in the absence (-▲-) and presence (-Δ-) of bacteriocin

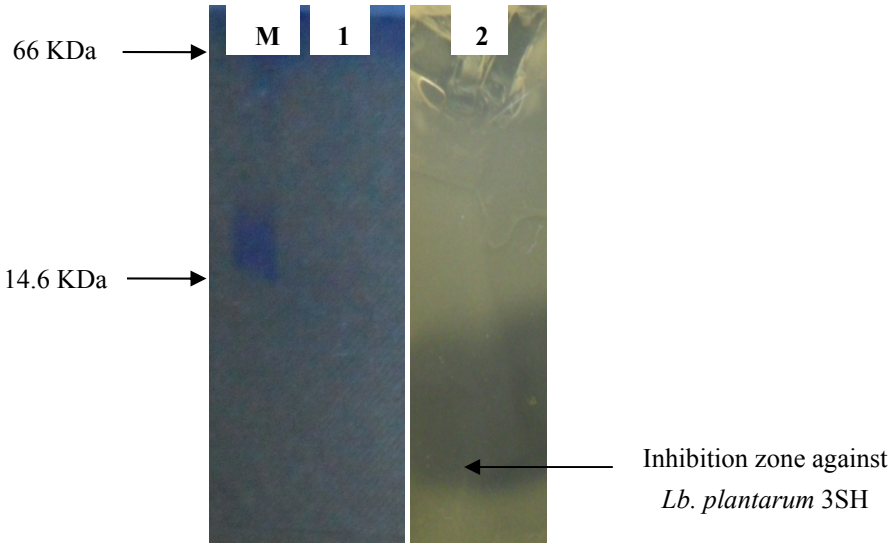

Figure 3. SDS-PAGE of bacteriocin Lp6SH. Lane M: Molecular weight markers (BSA and Lysozyme)

Lanes 1 and 2: Bac Lp6SH (Gel filtration active fraction). Inhibition zone indicates the position of the active peptide band. The gel was overlaid with viable cells of *Lb. plantarum* 3SH inoculated in MRS soft agar. Incubation was performed anaerobically at 30 °C for 24 h

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