

Study of Bacteriocin as a Food Preservative and the *L. acidophilus* Strain as Probiotic

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Abstract: Bacteriocin producing *Lactobacillus acidophilus* strain was isolated from the gut of marine prawn (*Penaeus monodon*). This bacteriocin has broad range of antibacterial activity against major food born pathogens. Maximum bacteriocin production was observed at temperature 50°C, pH 4 and 0.9% sodium chloride. The bacteriocin was purified by ammonium sulphate precipitate and ion exchange (DEAE cellulose) chromatography. Biochemically it was pure protein moiety and its molecular weight was 2.5 KDa. This study revealed the possibility of using bacteriocin as a food preservative and the *L. acidophilus* strain as probiotic.

Key words: Bacteriocin, chromatography, antagonistic activity, *L. acidophilus*

INTRODUCTION

The genus *Lactobacillus* is well diverse and consists of a number of different species with little commonality. They are gram-positive rods with a size range of 0.5-1.2×1-10 µm and non spore formers, producing lactic acid as a fermented end product (Aasen *et al.*, 2000). It includes over 25 species and the first level of differentiation is based on end-product composition. Some are homofermentative where as others are heterofermentative in nature. Lactic Acid Bacteria (LAB) are useful in the food industry. They reduce the pH in food, low enough to inhibit the growth of most of other microorganisms including common human pathogens, thus increasing the self life of food (Ivanova *et al.*, 2000). In the search for a food preservative, investigations on certain antibacterial proteins (bacteriocin) from lactic acid bacteria have been popular (Daeschel, 1990). Bacteriocin is proteinaceous compounds of bacterial origin that are lethal to bacteria other than the producing strain. Bacteriocin secreting microbes has selective advantage in a complex microbial niche. Generally, bacteriocins are named according to the genus or species of the strain that produces it. For example, plantaricin produced by *L. plantarum* (Joerger *et al.*, 2000). Bacteriocins produced by lactic acid bacteria have received considerable attention during recent years for their possible as biopreservative in food, with a resultant reduction in the use of chemical preservatives. *Lactiobacillus acidophilus* one of the most important lactic acid bacteria used for the production of fermented meat, grass and vegetable products (Ritz Barba *et al.*, 1991).

Several bacteriocin-producing strains have been isolated from marine habitat. The antimicrobial peptides produced by one of these strains, later identified as *L. acidophilus* (Van Reenen and Dicks, 1996), inhibits the growth of a number of food spoilage bacteria, this study reports on the spectrum of antibacterial activity,

production, characteristics and isolation of bacteriocin. The genetic relatness of this bacteriocin to bacteriocin is also discussed.

MATERIALS AND METHODS

Collection of samples: *L. acidophilus* strains were isolated using the method described by Todorov and Dicks (2004). *P. monodon* samples were collected from Mudasalodai area. The prawn gut samples were pooled and ground well, 1 g of pooled sample was taken for the study. They were serially diluted in sterile physiological saline and plated on MRS Agar, supplemented with 50 mg/L of Natomycin. The plates were incubated at 30°C for 72-96 h.

Identification: The strains were identified according to the method described by Michael (1981). The following tests were performed to observed pointing out the morphological, physiological and biochemical characteristics: The gas producing capacity was assessed by culturing the microorganisms on a medium containing peptone-15 g, casein-5 g, glucose-1 g, potassium acetate-0.2g, sodium thiosulphate-0.08 g, magnesium sulphate-0.05 g, agar-15 g and Tween 80-1 mL, which was incubated for 72 h at 30°C. Resistance to biliary salts was tested on a medium containing; tomato juice-10mL, peptone-1.5 g, glucose -2 g, NaCl-0.5 g, yeast extract-0.6 g, soluble starch-0.05 g, tauroglycocholate-2 g and Tween 80-0.1 mL. For pH assay, the cultures were grown on MRS broth at various pH ranges from, 3, 3.5, 4.5, 5.0, 5.5, 6.0 and 6.5. The fermenting capacity of various sugars was tested on a medium containing pepetone-10 g, NaCl-5 g, K₂PO₄-0.3 g and bromothymol blue (pH 6.5). The starch hydrolysis was performed on a medium containing: peptone-1.5 g, NaCl-0.5 g, yeast extract-0.6 g, galactose-0.5 g, starch-0.2 g, agar-2 g, Tween 80-0.1 mL (pH-6.5).

Detection of antibacterial activity: Antimicrobial activity was confirmed by using the agar spot test method as described by Eamanu *et al.* (2005). Seven mL of sterile BHI soft agar was cooled to 47°C and mixed with 10 µL of a cell suspension of bioassay strains (over night cultures). The soft agar was then poured over the agar plates and cooled at room temperature for 30 min. After the plates were solidified make 5 µL of culture free supernatant of test organism. The plates were incubated at 37°C for 18-24 h and examined for the presence of clear zone of inhibition of 2mm or more around the spot

Detection of bacteriocin titer: The titer of bacteriocin was quantified by the method described by Todorov and Dicks (2004). Cell free extract of *L. acidophilus* parallel dilution using physiological saline solution. Aliquots of 0.1 µL from each dilution were spotted in plates seeded with the bioassay strain (over night culture). The plates were incubated at 37°C for 18-24 h and examined for the presence of 2 mm wider clear zone of inhibition around the spot. The antimicrobial activity of bacteriocin was defined as the reciprocal of the highest dilution showed the inhibitory activity and it is expressed as Arbitrary Unit (AU).

Bacteriocin production and biomass in different media: The growth and bacteriocin production were measured as described by Eamanu *et al.* (2005). The test organism was inoculated into the MRS broth (Peptone-10.0 g/L, yeast extract-5.0 g/L, meat extract-5.0 g/L, glucose - 20.0 g/l, dipotassium phosphate - 2.0 g/l, dibasic ammonium citrate-2.0 g/L, sodium acetate-5.0 g/L, maganous sulphate-0.050 g/L, magnesium sulphate-0.010 g/L, tween 80-1ml/L (pH 6.5) and GP broth, glucose-2%, peptone-1%, CaCO₃-1% (pH 6.5). OD was measured at 600 nm for every one h regular interval and respectively evaluates the bacteriocin titer.

Inhibitory activity: The antimicrobial activity of bacteriocin was tested against the test organisms following the method described by (Todorov and Dicks, 2004). *L. acidophilus* inoculated into MRS broth and incubated at 30°C, without aeration until mid logarithmic phase of growth. Aliquot of 10 µL cell-free culture supernatant was spotted on the surface of agar plate seeded with actively growing cells of the test organism. Plates were incubated at the optimal growth temperature of the test organism.

- *Latobacillus bulgaricus*
- *Salmonella typhimurium*
- *Bacillus subtilis*
- *Staphylococcus aureus*
- *Salmonella paratyphi 'B'*
- *Escherichia coli*
- *Klebsiella sps*

- *Serratia marsacence*
- *Pseudomonas aeroginosa*
- *Vibrio cholerae*

Effect of physicochemical parameter on bacteriocin production: pH-Three hundred ml MRS broth was prepared and adjusted to pH 1, 2, 3, 4, 5 and 6, respectively with 6 M HCl or 6M NaOH and then autoclaved. Flask were inoculated with 2%v/v of 18h old culture of *L. acidophilus* and incubated at 30°C for 20 h, without aeration. The pH of the supernatants was adjusted to 6.0 with sterile NaOH, from which bacteriocin titer was assessed.

Temperature: Six Volumes of 300 mL of MRS broth were inoculated with 2% of 18 h old culture of *L. acidophilus* and incubated at different temperatures such as 10, 20, 30, 40, 50 and 60°C for 20 h, without aeration, from which bacteriocin titer was assessed.

Salinity: NaCl concentration from 0.1 to 1.0% was tested and bacterion titer was assessed as described by the (Todorov and Dicks, 2004).

Purification and characterization of bacteriocin

Ammonium sulphate precipitation: The crude bacteriocin sample was treated with ammonium sulphate as 10, 20, 30, 40, 50 and 60% saturation (Yang *et al.*, 1992). The precipitate was resuspended in 25mL of 0.05 M potassium phosphate buffer. The mixture was stirred for 24h at 4°C. Dialysis was followed in a tubular cellulose membrane (1000 cut off) against 2L distilled water for 24 h, from which the bacteriocin titer was performed.

Ion-exchange chromatography: The dialysate was used for purification by cation exchange column (DEAE cellulose column) and elution was performed by using a linear gradient from citrate phosphate buffer ranging from pH 2.6-7.0 (Macher and Klock, 1980). Protein content was determined by Bradford method (Sadasivam and Manickam, 1996). The bacteriocin titer was assessed.

Effect of enzyme and protease inhibitor: The methodology described by Todorov and Dicks (2004) was followed to assess the effect of enzyme such as á amylase, protease and catalase at the concentration of 0.1 and 1.0mg/mL on the bacteriocin activity.

Molecular size of bacteriocin-SDS PAGE: Molecular size of bacteriocin was determined using SDS PAGE gel following the procedure of Sambroo *et al.* (2006). Glass plates were assembled and 20 mL of 15% resolving gel was prepared and poured immediately to the notch

plate. It was overlaid with butanol, after polymerization was completed overlay was poured off and washed the top layer with deionized water. Then 8ml of stack gel was overlaid. Approximate volume of 1×SDS gel loading buffer and sample was taken. Heated it at 100°C for 3 min. Assembly was fixed in electrophoresis apparatus then 15 µL of sample and marker (2,500-40,000 KDa) was loaded respectively in the well, run the gel and stain with Coomassie brilliant blue.

RESULTS

LAB was isolated gut of *P. monodon* using MRS agar. The viable cell count of LAB was around 4.0×10^5 CFU/g. The isolated *Lactobacillus* strains show antagonistic activity against *L. bulgaricus*. The strains which showed the largest zone of growth inhibition was selected for further identification

The selected strain was identified as *L. acidophilus* based on its physiological and biochemical characteristic. The colonies were cream, beige, little sticks and smooth round colonies. The strain was gram positive rod. In liquid MRS broth it produced uniform turbidity. It was homofermentative and it showed positive reaction in the fermentation of galactose, glucose, fructose, mannitol, lactose, sucrose and maltose but not with rhamnose. It did not produce catalase and amylase. It was resistant to biliary salt and it produces H₂S.

Antagonistic activity was tested against ten major pathogens. Among the ten pathogens tested, all the human pathogens were found to be sensitive to bacteriocin except *Vibrio cholerae* (Table 1 and Fig. 6). Growth and bacteriocin production in MRS and GP broth Both MRS and GP broths, tested showed bacteriocin production at stationary phase at 14 h of incubation. In MRS broth 3400 AU/mL was produced by the strain where as in GP broth it produced only 800AU/mL (Fig. 1 and 2).

Production of bacteriocin at different pH, temperature and salinity: The bacteriocin activity was tested with different temperature and the activity was found to vary from 1600AU/mL-25000AU/mL, the maximum arbitrary unit was measured as 25600au/ml at temperature 40°C (Fig. 3). Regarding pH the maximum inhibitory activity was observed at pH 5.0 and minimum was observed at pH 1-3 (Fig. 4). Regarding various salinity (NaCl %) tested 0.9% NaCl was found to be suitable (Fig. 5).

Purification and characterization of bacteriocin Ion-exchange chromatography: The maximum inhibitory activity was measured at 50% saturation. In ion exchange chromatography (DEAE cellulose) the active fraction was eluted with pH 5.0-citrate phosphate buffer.

Table 1: Inhibitory activity of bacteriocin against ten food spoilage bacteria

Test organism	Result
<i>Bacillus subtilis</i>	(+)
<i>Staphylococcus aureus</i>	(+)
<i>Lactobacillus bulgaricus</i>	(+)
<i>Salmonella typhimurium</i>	(+)
<i>Salmonella paratyphi 'B'</i>	(+)
<i>Escherichia coli</i>	(+)
<i>Kelbsiella sp.</i>	(+)
<i>Serratia marcescens</i>	(+)
<i>Pseudomonas aeruginosa</i>	(+)
<i>Vibrio cholerae</i>	(-)

Positive: Diameter of zone >2 mm, Negative: Diameter of zone <2 mm

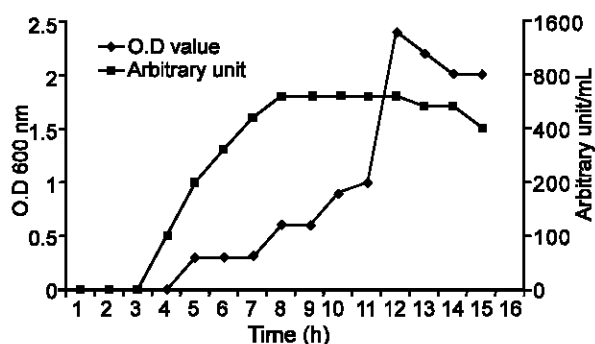


Fig. 1: Bacteriocin production in MRS broth

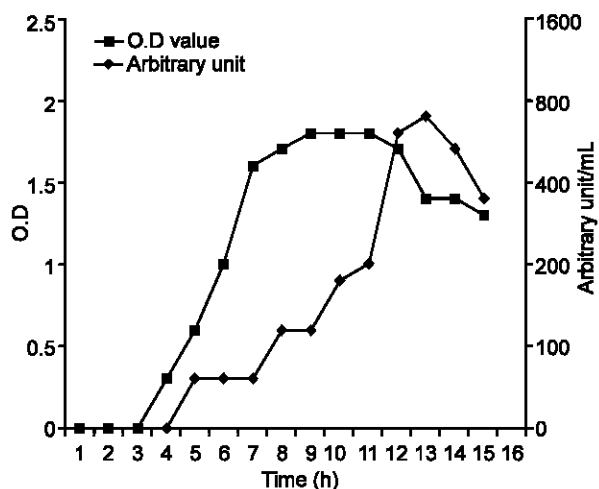


Fig. 2: Bacteriocin productions in GP broth

The molecular weight of the bacteriocin was determined as 2.5 KDa (Fig. 7).

Effect of enzyme on bacteriocin: When purified bacteriocin compound was treated with different enzymes, such as catalase, protenase K and α amylase, inactivation of inhibitory activity was observed only with protenase K (Table 2-4).

DISCUSSION

The present investigation highlights the isolation, partial characterization and activity of bacteriocin produced by *L. acidophilus*.

Prawn gut seems to be a good source of LAB. Present study revealed that *P. monodon* harbored LAB at the level of 4.0×10^6 CFU/gm in its gut, among strains tested; the most potential strain was selected and used for further study. The physicochemical characterization of the strain revealed that it was *L. acidophilus*.

It was tested against 10 different bacterial pathogens which are usually present in food and can cause food borne illnesses in human being. The bacteria selected were *L. bulgaricus*, *Salmonella typhimurium*, *E. coli*, *Bacill subtilis*, *Staphylococcus aureus*, *Salmonella paratyphi 'B'*, *E. coli*, *Klebsiella sp.*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Vibrio cholerae*. The result indicated the present strain

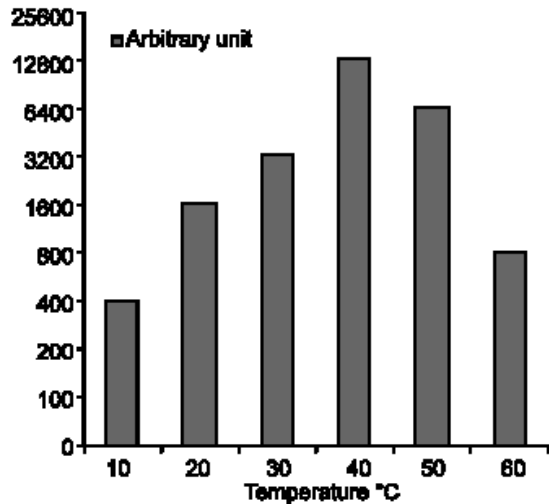


Fig. 3: Bacteriocin production in different temperatures

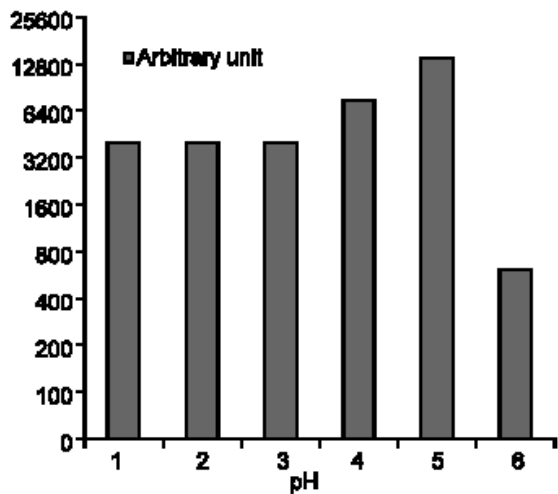


Fig. 4: Bacteriocin production in Different pH

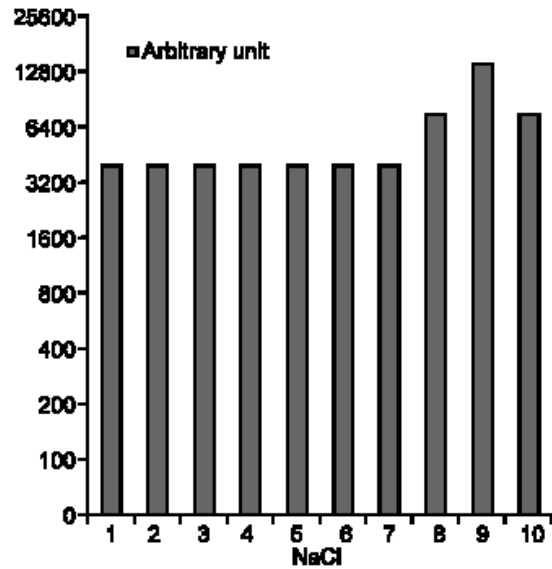


Fig. 5: Bacteriocin production in different salinity

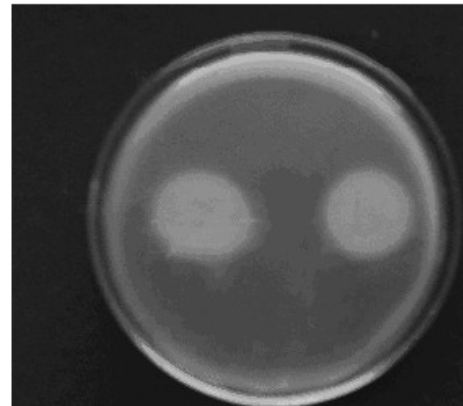


Fig. 6: Inhibitory activity of bacteriocin

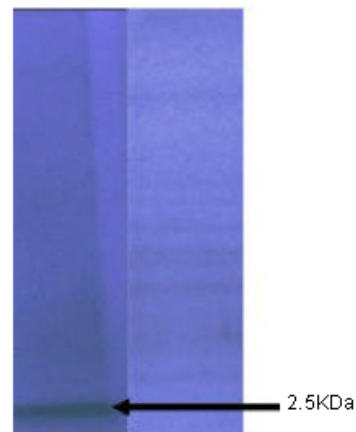


Fig. 7: SDS GE: Purified protein (bacteriocin) was about 2.5KDa

Table 2: Bacteriocin treated with protenase K

Protenase K mg/mL /dilution factor	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	1:51200
0.1	+	+	+	+	-	-	-	-	-	-
1	+	-	-	-	-	-	-	-	-	-

Table 3: Bacteriocin treated with α amylase

α amylase mg/mL /dilution factor	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	1:51200
0.1	+	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+	+	+

Table 4: Bacteriocin treated with catalase

Catalase mg/mL /dilution factor	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	1:51200
0.1	+	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+	+	+

seemed to have antagonistic activity against nine pathogens in the order of *L. bulgaricus*, *Salmonella typhimurium*, *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella paratyphi 'B'*, *E. coli*, *Klebsiella* sp., *Serratia marcescens*, *Pseudomonas aeruginosa*. The study had proved the possibility of using this strain as a biopreservative or a probiotic.

Bacteriacin are antimicrobial agents produced by bacteria which are active against closely related bacteria as claimed by Klaenhammer (1993). They have been proved active against many other bacteria also including pathogens described by Flythe and Russell (2004). Hence they may be used as probiotic or as biopreservative especially in the acid fermentation of food. In the present study an attempt was made to study the bacteriocin produced by a prawn gut borne *Lactobacillus* sp and the bacteriocin production will be studied in further. Further studies is needed on production of bacteriocin.

Bacteriocin production was strongly dependent on pH, nutrient source and incubation temperature as claimed by Todorov and Dicks (2004). Various physicochemical factors seemed to affect bacteriocin production as well as its activity. Maximum activity was noted at pH 5, temperature 40°C and 0.9% NaCl. From the results proved that it can be used in acidic foods like pickle, yogurt etc as the optimum pH for activity was found to be pH 4.0. It might be secondary metabolites. MRS seemed to be more suitable medium compared to GP broth for the bacteriocin production.

The bacteriocin seemed to be a pure protein of about 2.5 KDa, since it was found to be active at pH 5, it deserves further study specially the molecular aspects of it. As maximum inhibitory activity was found in the culture medium at the stationary phase.

Bacteriocin is a bacterial substance, which are has biological protein moiety and a bactericidal mode of action against the homologues species (Yang *et al.*, 1992). Chemical analysis indicated that some bacteriocin, Examble; plantaricin, are quite complex molecules and lipid and carbohydrate components in

addition to protein with lipid are simple proteins. Bacteriocin of LAB is particularly important because of the essential role of the bacteria in the majority of fermented foods. The present study also showed that the bacteriocin is the unfairly protein moiety and their molecular weight of the protein was defected about 2.5 KDa protein.

Nisin is the first use as a food preservative agent in 1931. Nisin have first received approval by food and drug administration (FDA) to be use in pasteurized processed cheese in 1988 as prescribed by Rosland *et al.* (2005). Like nisin the bacteriocin produced by *L. acidophilus* in the present study also has the potential to develop probiotics and biopreservative.

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