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Research Article

Isolation, Identification and Characterization of Bacteriocin from Lactobacillus Lactis

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Abstract

Many consumers today are concerned about the synthetic chemicals used as preservatives in food, and there is a resulting trend towards less processed food. Bacteriocin produced by *Lactobacillus lactis* strain isolated from the marine environment, showed broad range of antibacterial activity against some major food borne pathogens. Sediment samples were collected at Parangipettai coast and stored in the laboratory at 4ºC for used to isolate the *Lactobacillus* spp. The samples were diluted and plated on the de Rogosa and Sharpe agar and The isolated strain was grown in MRS broth (pH-6.0) seeded with 5% inoculum of overnight culture and maintained anaerobically at 30ºC for 48 h. After incubation, cells were removed from the growth medium by centrifugation. The cell-free supernatant was adjusted to pH 6.0 using 1N NaOH and it was used as crude bacteriocin (14). Maximum bacteriocin production was observed at 30°C, pH 6.0 and 1.5% sodium chloride solution. The enzymes - amylase, DNase, RNase and lipase had a positive effect on bacteriocin production. Proteinase K and pepsin strongly inhibited bacteriocin production. Among detergents, Sodium dodecyl sulphate (SDS), Tween 80 and Tritone X-100 stimulated bacteriocin production. EDTA and urea strongly inhibited bacteriocin production. The molecular weight was 94 kDA. Therefore, the peculiar antimicrobial characteristics of *L. lactis* can positively have impact on their use as starter cultures for traditional fermented foods, with a view to improving the hygiene and safety of the food products so produced.

Keywords: Bacteriocin, amylase, DNase, RNase, lipase, antibacterial activity.

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Introduction

Many consumers today are concerned about the synthetic chemicals used as preservatives in food, and there is a resulting trend towards less processed food. Alternatives include vacuum packing and refrigeration of foods. However, there are concerns among the regulatory agencies and food processors that psychrotrophic food-borne pathogens and food-spoilage organisms, even when initially present in low numbers, can multiply during extended storage and make these foods unfit and unsafe for consumption under refrigeration and without oxygen [1]. Many Gram-Positive bacteria, Particulary many lactic acid bacteria (LAB) are known to secrete ribosomally-synthesized peptides or portins that have antimicrobial activity, theses componds (bacteriocin) have been shown to display inhibitory activity against closely related bacteria[2]. Bacteriocin are antibacerial proteinous substances or peptides, which are produced by various bacteria for the inhibition of growth and destruction of closely related bacterial strains [3]. These compounds show great promise and are attractive candidates for use as biopreservatives in the food industry[4]. Research on LAB bacteriocin production, purification, genetics and applications is burgeoning [5]. Most bacteriocin produced by Gram-positive bacteria are from lactic acid bacteria [6,7]. They are also relatively hydrophobic and heat stable [8]. The marine environment is rich in nutrient and organic matter. Hence the strain *Lactobacillus lactis* was isolated from the marine environment. In this paper we characterized the bacteriocin produced by L.lactis, the optimum conditions for bacteriocin production, estimated the molecular weight of the bacteriocin.

Materials and Methods

Sample collection

Sediment samples were collected during July, 2013 at Parangipettai coast (Lat:11°30'N, Long:79°46'E) in the Bay of Bengal (Tamilnadu). The sediment samples were stored in the laboratory at 4ºC in sterile specimen cups until they were used to isolate the *Lactobacillus* spp. All the experiment was conducted by Center of Advanced Study in Marine Biology, Annamalai University.

Isolation and identification

Dilutions (10G1 - 10G6) of one gram of sediments in sterile 50% aged seawater were prepared and plated on the Man Rogosa agar (MRS agar) medium (Hi Media Laboratory Pvt. Ltd. Mumbai, India) to isolate the *Lactobacillus* [9]. The strains were sub-cultured onto MRS agar slant (medium with 50% sea water), incubated at 30 ºC for 24 h and were preserved in 20% glycerol at -80 ºC. One of the isolate was selected for further studies which exhibited strong inhibitory activity against indicator strains and identified on the basis of growth, cell morphology, and gram staining and catalase activity. Further, identification of the species of these *Lactobacilli* was performed according to carbohydrate fermentation patterns and growth at 15 and 45ºC in the de Man Rogosa Sharpe (MRS) broth as described in Bergey's Manual of systematic bacteriology [10].

Production of crude bacteriocin

The isolated strain was grown in MRS broth (Hi Media Laboratory Pvt Ltd. India) (pH-6.0) seeded with 5% inoculum of overnight culture and maintained anaerobically at 30ºC for 48 h. After incubation, cells were removed from the growth medium by centrifugation (10,000×g for 15 min, 4ºC). The cell-free supernatant was adjusted to pH 6.0 using 1N NaOH and it was used as crude bacteriocin [11].

Bacteriocin assay

The antibacterial spectrum of the bacteriocin from *Lactobacillus lactis* was determined using the well diffusion method. The supernatant from a 48-h culture of *Lactobacillus lactis* was filter sterilized

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by passage through a 0.45 µm pore size membrane filter (PALL Corporation, Mumbai). Aliquots (50 µl) of the sterile supernatant were placed in 4-mm-diameter wells that had been cut in Mueller-Hinton agar plates previously seeded with the indicator bacteria. After 12-18 h of incubation, the diameters of the zones of growth inhibition were measured. Antimicrobial activity was expressed in arbitrary units (AU/ml). One AU was defined as the reciprocal of the highest level of dilution resulting in a clear zone of growth inhibition [12]. (i.e. turbidity ≤50% of the turbidity of control culture grown without *L. lactis* supernatant).

Optimization of culture conditions

The selected strain *Lactobacillus lactis* was subjected to different culture conditions to derive the optimum conditions for bacteriocin production. Growth and bacteriocin production were estimated at various temperatures (20, 25, 30, 35, 40 and 45ºC), pH (4.0, 5.0, 6.0, 7.0, and 8.0)

Purification of bacteriocin

The crude bacteriocin was precipitated with 80% ammonium sulphate (Ranbaxy, New Delhi) saturation. The precipitate was dialysed against 20 mM potassium phosphate buffer (pH 7.0) for 12 h at 4ºC. Further purification was carried out in ion exchange chromatography (DEAE-Cellulose, Hi Media Laboratory Pvt Ltd. India). The dialyzed protein was applied to a DEAE- Cellulose A-50 column (20 mm diameter×60 mm long), pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.0). After washing the column with 3 vol. of equilibration buffer, bound proteins were eluted stepwise using phosphate buffers of increasing molarity and decreasing pH values at room temperature (approx. 25 ºC). The flow rate was adjusted to 24 ml h^{-1} and fractions (1 ml each) were collected. The fractions showing high bacteriocin activity were pooled and concentrated in lyophilizer.

Determination of protein:

Protein concentration of the bacteriocin in supernatant was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

Molecular weight determination in SDS-PAGE:

The molecular weight of the bacteriocin was determined by 15% Sodium dodecylsulfate polyacrylamide gel electrophoresis,[13]. in LKB Bromma 2050 Midget electrophoresis units (Pharmacia Amersham Co). After electrophoresis, the gel was stained with Comassie Brilliant Blue R-250. Range moleculer markers (29-200 kDa) with five polypeptides were used as a marker.

Result

Isolation and identification of bacterial strain

The bacteriocin producing strain was isolated from the marine environment of the Parangipettai and the selected strain was identified as *L. lactis* based on its physiological and biochemical characteristic (Table 1).

Determination of inhibitory spectrum

The susceptibilities of various Gram-positive and Gram negative bacteria to growth inhibition by the supernatant of *L. lactis* are presented in Table 2. It shows inhibitory activity against *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella shiga* and *Shigella boydii*. Among these, maximum activity observed against *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* and minimum activity observed against *Shigella boydii* (Fig. 1 and 2).

Effect of temperature, pH and salt concentration on bacteriocin activity

Temperature and pH played an important role in cell growth as well as bacteriocin production. The bacteriocin activity was tested with different temperatures (20, 25, 30, 35, 40 and 45ºC). Furthermore, the maximum arbitrary unit was measured as 2647 AU/ml at 30ºC and minimum levels were recorded as 1876 AU/ml at 20ºC (Fig.

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3). Regarding pH the maximum arbitrary unit was measured as 2847 AU/ml at pH 6.0 and minimum in 1969 AU/ml at pH 9.0(Fig.4.).

Purification of bacteriocin

In the purification of filtrate culture, was removed by centrifugation, and the proteins were concentrated by 80% ammonium sulphate precipitation and dialysis. The recovered proteins were then fractionated by ion-exchange chromatography, using DEAE- Cellulose. All procedures were done in cold room. Extracellular bacteriocin was purified up to 11.11 fold from culture supernatant. The overall yield and activity are summarized in Table 3.

Molecular weight determination in SDS-PAGE

Molecular weight of the bacteriocin was determined by SDS-PAGE gel electrophoresis (Fig.5.). Single protein band was observed when stained with Comassie blue and it clearly indicated the purity of the protein. The molecular weight of the purified bacteriocin was calculated to be about 94 kDa.

Table 1: Physiological an d biochemical characteristic

Physiological and biochemical characteristic	Result
Colony morphology	Creamy, little sticks and smooth round colonies
Gram staining	Gram positive, rod
Growth in MRS broth	uniform turbidity
Type of fermentation	Homo fermentative
Galactose, glucose, fructose, mannitol, lactose, sucrose an maltose	Fermentation positive
Catalase, oxidase, indole and amylase production	Negative
Growth in Bilary salt	Resistant
H_2S production	Positive

Table 2: Antibacterial activity of *Lactobacillus lactis* against human pathogen

S.no.	Human Pathogen	Zone of Inhibition level (mm)
1	Staphylocous aureus	4
2	Bacilus megaterium	2
3	Bacilus subtilis	5
4	Enterococcus faecalis	4
5	Escherichia coli	2
6	Pseudomonas aeruginosa	3
7	Shigella shiga	2
8	Shigella boydii	1

***Low activity (mm). moderate activity (2.5mm) - no activity**

Table.3. Summary of the purification steps of bacteriocin from the culture supernatant of *Lactobacillus lactis*.

Purification Stage	Volume (ml)	Total activity (AU/ml)	Total protein (mg)	Specific activity (AU/mg)	Purification (fold)	Recovery (%)
Culture supernatant	100	7350	225.2	32.64	0.0	100
Ammonium sulphat precipitation, (80% saturation and dialysis	25	2680	28.7	105.92	3.25	41.36
DEAE-Cellulose chromatography	5	800	0.68	1176.47	11.11	26.32

Fig. 1. *Lactobacillus lactis* showing bacteriocin activity by agar-well diffusion method against *Enterococcus faecalis* as sensitive culture

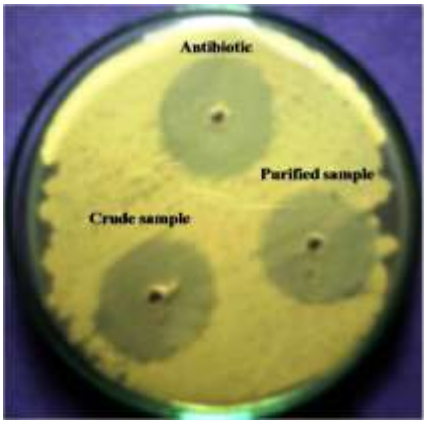

Fig. 2. *Lactobacillus lactis* showing bacteriocin activity by agar-well diffusion method against *Staphylococcus aureus* as sensitive culture

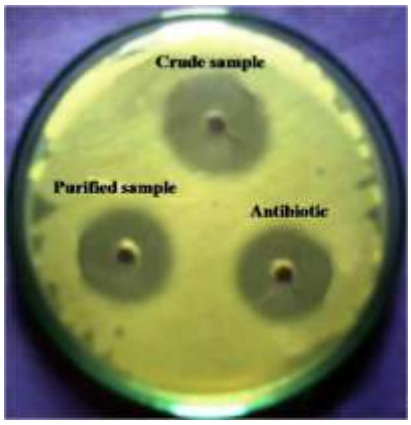

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Fig. 3. Effect of temperature on bacteriocin production

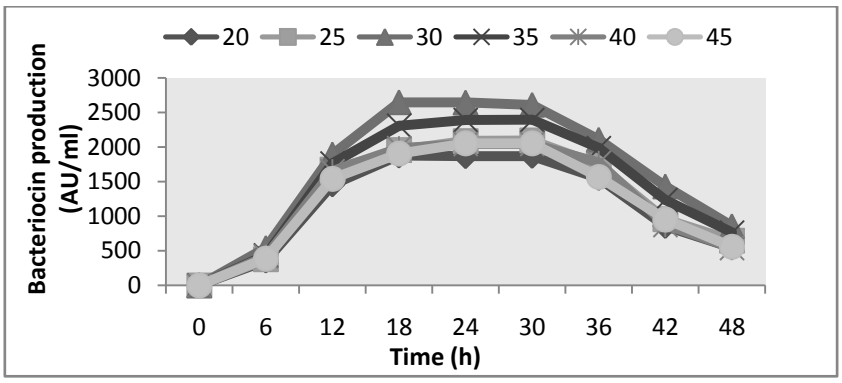


Fig. 4. Effect of pH on bacteriocin production

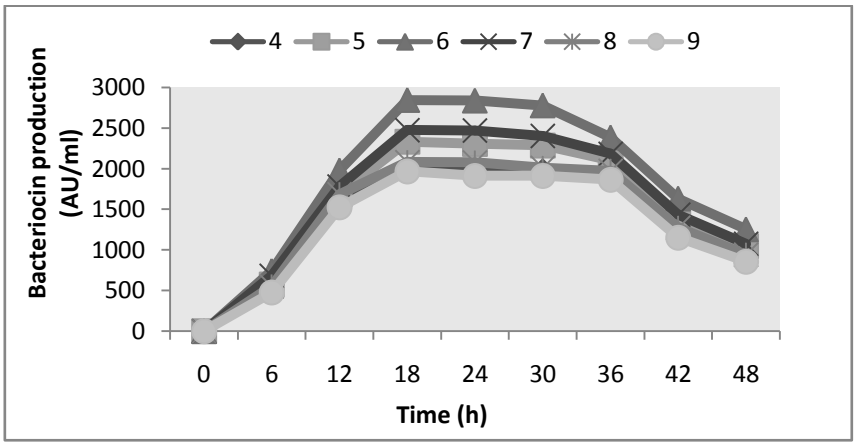
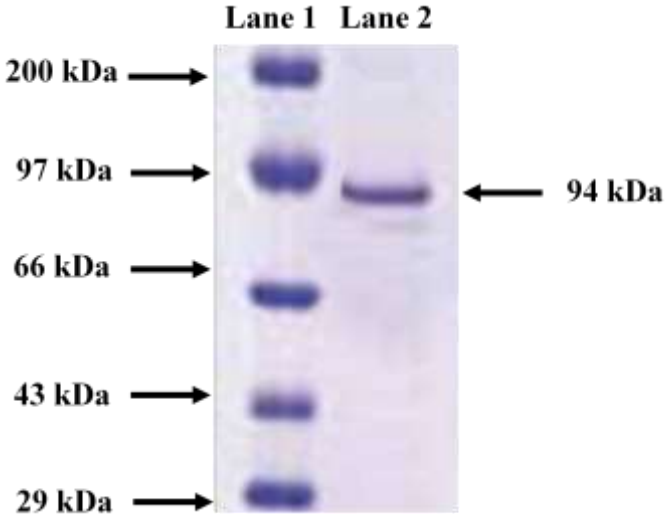


Fig. 5. SDS-PAGE of the purified enzymes: Lane 1, molecular weight markers; lane 2, Bacteriocin.



Discussion

Microbes produce an extraordinary array of defense systems. These include broad-spectrum classical antibiotics, metabolic by-products such as the lactic acids produced by lactobacilli, lytic agents such as lysozymes, numerous types of protein exotoxins and bacteriocins. The microbial weapon of choice, as measured by abundance and diversity in natural populations, is the heterogeneous family of proteins known as bacteriocins[14]. Bacteriocins are loosely defined as biologically active protein moieties with a bacteriocidal mode of action [15]. They differ from traditional antibiotics in one critical way; they have a relatively narrow killing spectrum and are often only toxic to bacteria closely related to the producing strain.

The present investigation highlights the isolation, characterization and activity of bacteriocin produced by *L. lactis* from marine environment. It is rich in nutrient and organic matter. To state that the isolate *L. lactis* was tested for antibacterial activity against gram-positive and gram-negative bacteria such as *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus cereus*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella shiga*, *Shigella dysenteriae* and *Shigella boydii* associated with food borne illnesses. The highest inhibitory activity was demonstrated against *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* while the least activity was demonstrated against *Shigella boydii*. The inhibitory effect demonstrated by *L. lactis* against these bacteria is an indication of possession of antibacterial activity. Results also revealed the presence of the compound bacteriocin in the test organisms. Bacteriocins have been reported to be inhibitory against several other bacteria [16,17]. Possession of bacteriocin by *L. lactis* is an indication that the bacteria can be used as probiotic and as biopreservative. Pal *et al.* [2005] reported twenty five colonies of LAB were isolated and screened for bacteriocin production from traditional fermented food dosa. Ten isolates showed good antimicrobial activity against Gram-positive viz., *Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes* as well as Gram-negative viz., *Pseudomonas aeruginosa, Vibrio parahaemolyticus* and *Aeromonas hydrophila* microorganisms.

Bacteriocin production was strongly dependent on pH, nutrients source and 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 6.0, temperature 30^{o}C and 1.5% NaCl. From the results proved that it could be used in acidic foods like pickle or yogurt. It might be secondary metabolites. MRs seemed to be more suitable medium for the bacteriocin production. Similar results were observed by [20].

Purified bacteriocin from *L. lactis* revealed homogeneity of a single protein band on 15% native PAGE. Its molecular weight was estimated at 94 kDa by SDS-PAGE. Similar results were recorded by [21,22]. In conclusion therefore, the peculiar antimicrobial

characteristics of *L. lactis* can positively have impact on their use as starter cultures for traditional fermented foods, with a view to improving the hygiene and safety of the food products so produced.

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