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Research Article

ISOLATION AND IDENDIFICATION OF LACTIC ACID BACTERIA FROM FERMENTED PRODUCTS AND ITS SPECTRUM OF INHIBITORY ACTIVITY

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Abstract: Lactic acid bacteria (LAB) were isolated from raw and fermented products like milk, curd, batter and pickle. Out of 44 isolates, 16 species were identified. Among them*Pseudomonas aeruginosaBacillus cereus , Staphylococcus aureus, , Klebsiella pneumonia*were dominant.Bacteriocin like inhibitory substance (BLIS) from the four isolates were tested against selected pathogens of both gram positive and gram negativegroup. The proteins were fractionated by ion-exchange chromatography, using DEAE- Cellulose .The Extracellular bacteriocin was purified from culture supernatant.The overall yield and activity was maximum in the concentration of 80%.and the activity was inhibited by proteinase K, however it remained resistant to other enzymes including protease activity in the culture supernatant shows 14mm, Ammonium sulphateprecipitatation80 % shows 16 and DEAE-Cellulose-Chromatography purification shows the maximum activity of 21mm.The molecular weight of the purified bacteriocin was calculated to be about 31.0kDa.
Key words: Bacteriocin, Lactic acid bacteria, Antimicrobial, AgarWell diffusion assay

INTRODUCTION:

In the production of food, it is crucial to take proper measures for ensuring its safety and stability during the shelf-life. Food preservation is carried out to maintain the quality of raw material and physiochemical properties as well as functional quality of the product providing safe and stable products. Nowadays, extensive work has been carried out on bacteriocins and bacteriocin producing strains of Lactic acid bacteria (LAB) for their potential use as biopreservatives.[1] Bacteriocins are the most abundant of antimicrobial compounds produced by bacteria and are found in all major phylogenetic bacterial lineages.[2] Generally, these substances antagonize only those bacterial species that occupy the same ecological niche or closely related to the producer organism. Many phytopathogenic bacteria, including members of the *Corynebacterium, Erwinia, Pseudomonads* and *Xanthomonas*produce proteinaceousbacteriocins.[3]

Some members of LAB produce bacteriocins and bacteriocins-like substances which may inhibit growth of spoilage and pathogenic microorganisms. Bacteriocins from LAB are bioactive peptides or proteins with antimicrobial activity toward Gram positive bacteria, including closely related strains and/or spoilage and pathogenic bacteria .Bacteriocins are ribosomaly synthesized and extracellulary released bioactive peptides or peptide complexes which have bactericidal or bacteriostatic effect. Use of either the bacteriocins or the bacteriocin-producing LAB like starter cultures for food preservation has received a special attention. Moreover, bacteriocins are innocuous due to proteolytic degradation in the gastrointestinal tract .*S. thermophilus* is a lactic acid bacterium of major importance in food industry like the manufacture of yoghurt.

Materials and Methods

Microorganisms

The indicator organisms namely *Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia* were isolated from fermented vegetables

Screening of LAB for antimicrobial activity

Milk, curd, and pickle were procuredfrom market in Salem, Tamilnadu. MRS agar andbroth (HiMedia, Mumbai, India) were used forenumeration and culture of LAB .

LAB were isolated on MRS agar and incubated anaerobically at 37 ^{o}C for 2-3 days. Five well isolated colonies were picked up and transferred to MRS broth. They were propagated twice and streaked on MRS agar to check the purity of the isolates and then stored in MRS soft agar (0.5 %) overlaid with 50 % glycerol at -20 ^{o}C.

These isolates from appambatter were tested for their ability to produce bacteriocins. These were inoculated into MRS broth and incubated at 37 ^{o}C for 48 h. Cell free supernatants adjusted to pH 5.0 with 2 N NaOH, were concentrated to one tenth of the original volume by flash evaporator, sterilized by passing through 0.22 µm membrane filter (Millipore, India) and evaluated for antimicrobial activity by agar well diffusion method

The cultures were identified according to their different morphological, cultural, physiological and biochemical characteristics. The purified bacterial culture was subjected to a range of biochemical tests for identification

Preparation of crude bacteriocins:
The strain that was selected as potential bacteriocin producer was grown in MRS broth at 29°C for 24 hours. Cells were separated by centrifugation (6000g, 30 min, 4°C). The cell free supernatant (CFS) and was maintained at pH 7.0. Bacteriocin activity in the supernatant was then tested by agar well diffusion assay was further subjected.Effect of temperature, Effect of Heat, Effect of pH and Ammonium sulphate precipitation.[4]

Effect of temperature:
In order to test the heat resistance, 10 ml of partially purified bacteriocin preparation was heated for 15 minutes at 60°C, 70°C, 80°C, 100°C and 121°C (pressure, 15 psi) respectively. Residual bacteriocin activity was detected against food pathogens *B. cereus, St. aureus, , Ps. aeruginosa, klebsiella pneumonia* at each of these temperatures by agar-well diffusion assay.

Effect of Heat on bacteriocin activity
Samples of crude bacteriocin were used for these tests. Aliquots of the semi-purified bacteriocin were exposed to heat treatments of 40°C for 40 min, 65°C for 40 min, 95°C for 20 min, and 121°C for 20 min, and then were tested for remaining antimicrobial activity.

Effect of pH on bacteriocin activity:
According to the method described by Karaoglu*et al.* sensitivity of partially purified bacteriocin preparation to different pH values was tested by adjusting the pH of the bacteriocin in the range of pH 4 to 9 with sterile 1N NaOH and 1N HCl. After 2 hours of incubation at room temperature, residual activity of each of the samples was determined against the indicator organism by agar-well diffusion assay.

Ammonium sulphate precipitation:
The cell-free supernatant was used as starting material for protein precipitation. Ammonium sulphate was gradually added to a final concentration (w/v) of 40%, 60%, 70% and 80% respectively and agitation continued for overnight at 4°C. The precipitate was collected (at every step) by centrifugation at 6000g for 45 min., and redissolved in 20 ml of nutrient broth (pH 7.0) and assayed for maximal bacteriocin activity.

Bacteriocin activity units:
Serial dilutions of cell free neutralized supernatant of the producer strain and of partially purified (80% ammonium sulphate precipitated) bacteriocin were prepared and followed for agar well diffusion assay. Plates were incubated overnight at 29°C and zones of inhibition around each well were measured in mm. The bacteriocin titer was expressed as arbitrary or activity unit/ml. One arbitrary unit (AU) of bacteriocin is defined as the reciprocal of the last serial dilution demonstrating significant inhibitory activity.

$$AU/ml = \frac{\text{Reciprocal of the highest dilution}}{\text{Volume of bacteriocin added}} \times 100$$

Effect of proteolytic enzymes on bacteriocin activity:
Action of proteolytic enzymes was tested on partially purified bacteriocin preparation by treatment with protease (Bacterial source), proteinase K at a final concentration of 1mg per ml. It was then incubated at room temperature for 2 hours and residual activity of bacteriocin was assayed along with bacteriocin.

Partial Purification OF Bacteriocinand Molecular Weight Determination of bacteriocin was determined by SDS-PAGE gel electrophoresis.

RESULT :
Based on morphological and biochemical tests, all the isolates were identified as belonging to lactic acid bacteria (LAB) group which was identified as Lactobacillus sp. The isolate (giving maximum antimicrobial activity) was Gram-positive, rod shaped, negative for catalase and peroxidase test, having circular and white colonies on the MRS media. The strain was also positive for mannitol, sucrose, glucose, and lactose, negative for citrate which was shown in the table 1.

Biomass andbacteriocin production:
Measurement of biomass and bacteriocin productions. Results showed that *Lactobacillus sp produced*bacteriocin in MRS broth. The strain *Lactobacillus sp exhibited* a good bacteriocin activity with the higher concentration of 100µl against *Pseudomonas aeruginosa,Klebsiella pneumonia, Staphylococcus aureus, Bacillus cereus*; the activity shows maximum results at pH**7**, temperature **80 °C** andEffect of Heat on bacteriocin activity **90°C -20 min**.

Effect of temperature, pH and Effect of Heat on bacteriocin activityon bacteriocin activity:
Temperature and pH played animportant role in cell growth as well as bacteriocinproduction. The bacteriocin activity was tested withdifferent temperatures (60, 70, 80 and 100). Regarding pH themaximum was measured at pH 7

Purification of bacteriocin:
In the purification of filtrate culture, was removed by centrifugation, and the proteins were concentrated by various concentration 40%, 60%, 70%, and 80% ammonium sulphate precipitation. The recovered proteins were then fractionated by ion-exchange chromatography, using DEAE- Cellulose. Extracellular bacteriocin was purified up to 11.11 fold from culture supernatant. The overall yield and activity was maximum in the concentration of 80%.and the activity was inhibited by proteinase K, however it remained resistant to other enzymes including protease activity in the culture supernatant shows 14mm, Ammonium sulphateprecipitatation80 % shows 16 and DEAE-Cellulose-Chromatography purification shows the maximum activity of 21mm

Molecular weight determination in SDS-PAGE:
Molecular weight of the bacteriocin was determined by SDS-PAGE gel electrophoresis. 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 31.0kDa.

Table-1: Biochemical and morphological characterization

S. No	Tests	*Lactobacillus sp*
1	Gram's Staining	+
2	Motility test	-
3	Indole Test	-
4	Methyl red Test	-
5	VP Test	-
6	Citrate Utilization Test	+
7	Urease Test	_
8	Starch hydrolases	+
9	Casein Hydrolases	+
10	Gelatin Hydrolases	-
11	Nitrate reduction Test	-
12	Oxidase Test	-
13	Catalase Test	-
14	Glucose Test	A
15	Lactose Test	A
16	Sucrose Test	A
17	Mannitol Test	A

(+ Positive, - Negative, A-Acid Production, NA-No Gas production ,W- Weak , G-Gas production. The identified biochemical and morphological of organism*Lactobacillus sp*

Table: 2: Effect of different temperature&pH bacteriocin activity against Clinical Pathogens

Organisms	zone of inhibition (T°) (mm)				zone of inhibition(pH) (mm)			
	60	**70**	**80**	**100**	**5**	**6**	**7**	**8**
Pseudomonas aeruginosa,	11	12	14	11	12.5	14.2	15	13
Klebsiella pneumonia	13	12	13	12	12	14.8	16.4	14
Staphylococcus aureus	14	14	12	12	13	15.4	16	13
Bacillus cereus	12	13	15	11	14	14	17.2	12

Table: 3: Effect of Heat on bacteriocin activity against Clinical Pathogens

organisms	zone of inhibition (mm)			
	40°C- 40 min	**65°C-40 min**	**90°C -20 min**	**121°C-20min**
Pseudomonas aeruginosa,	10	14	16	13
Klebsiella pneumonia	11	14	16	12
Staphylococcus aureus	12	15	15	10
Bacillus cereus	12	16	15.6	12

Table: 4: Antimicrobial Activity against Clinical PathogensVS Antimicrobial Activity against Clinical Pathogens(Ammonium sulphate precipitation)

organisms	zone of inhibition (µl) (mm)				zone of inhibition (mm)			
	25 µl	50 µl	75 µl	100 µl	40%	60%	70%	80%
Pseudomonas Aeruginosa	7	8	9	10	12	12.5	9	12
Klebsiella pneumonia	7	8	12	14	13	14	14.8	16
Staphylococcus aureus	7	8	10	12	14	13	14	14.3
Bacillus cereus	8	10	10	12	14.5	16	16.5	18

Table: 5 Partial purification of Bacteriocin from culture supernatant of Lactobacillus sp Bacteriocin activity units

Sample	Volume (ml)	Activity (au/ml)
Culture supernatant	500	120
Ammonium sulphateprecipitatation80 %	100	60

Table: 6 Effect of proteolytic enzymes on bacteriocin activity

Sample	Activity in mm
Culture supernatant	14
Ammonium sulphateprecipitatation80 %	16
DEAE-Cellulose-Chromotography purification	21

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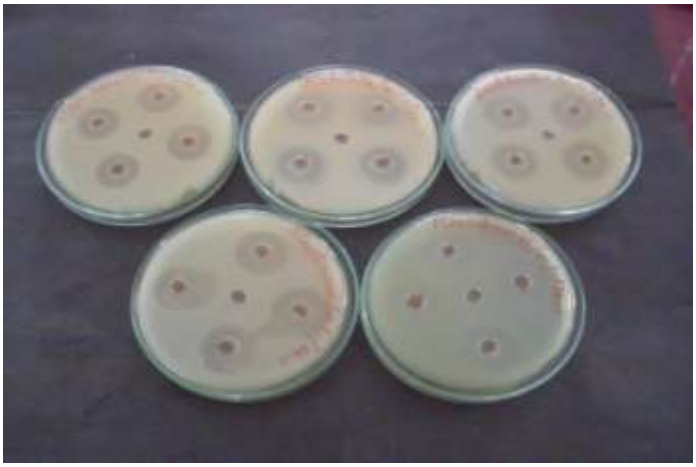
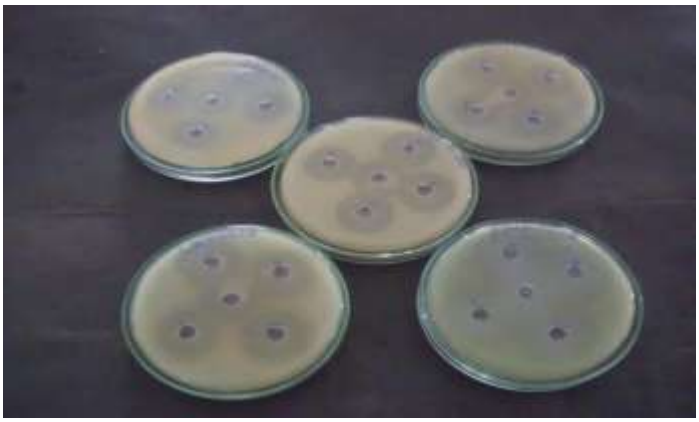
Picture: 1 The Picture Showing the Effect of temperature on bacteriocin activity

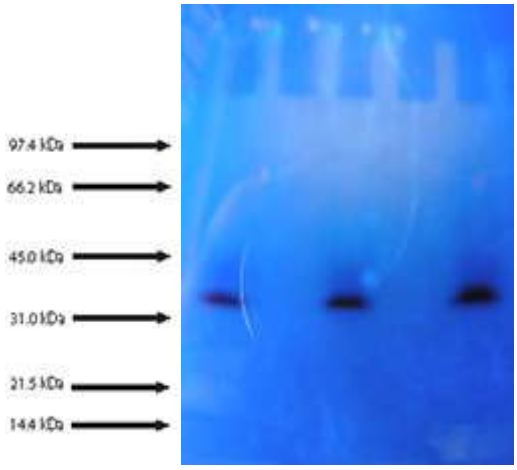


Picture: 2 The Picture Showing the Effect of pH on bacteriocin activity



Picture: 3 The Picture Showing the Effect of Heat on bacteriocin activity

Picture: 4 The Picture Showing the Effect of purification on bacteriocin activity



Picture: 13 The Picture Showing the SDS PAGE

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