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Purification and Optimization of Stability of Bacteriocin by *Lactobacillus* Species Isolated from the Curd Sample

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Abstract: **- Bacteriocins are small ribosomally synthesized antimicrobial peptides, that is produced by some microorganisms including lactic acid bacteria (LAB), a group of Gram-positive bacteria (cocci, rods) expressing high tolerance for wide range of pH. The bacteriocin shows broad range of thermo stability from 37°C to 121°C for 30 minutes. The well diffusion assay showed the bacteriocin produced by LAB is inhibiting the growth of Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Bacillus subtilis presenting a wide spectrum of inhibition. The molecular weight of bacteriocin is 6 kDa and it was determined by SDS-PAGE. This bacteriocins kill bacteria rapidly and are biologically active at very low concentrations. There is many bacteriocins have been investigated with respect to their potential use in promoting human, plant, and animal health, and also, as food biopreservatives. Bacteriocins produced by LAB are particularly interesting since several LAB have been granted as GRAS (Generally Recognized as Safe) status from FDA. Because it is not always possible to extract active bacteriocins secreted from cells grown in liquid medium, we developed a simple and inexpensive extraction procedure, using ammonium sulphate precipitation and DEAE Sephadex A-25 Column. By using Folin's Lowery method, the concentration can be finding out. I hereby present a detailed procedure that leads to the rapid extraction of secreted bioactive bacteriocin peptides from the Lactic acid bacteria, isolated from curd sample. Also present a simple method for the optimization of bacteriocin for their detection of activity from the purified bacteriocin sample.**

Keywords: Bacteriocin, Lactic acid bacteria, DEAE Sephadex A-25 Column, Ammonium sulphate precipitation, Bacteriocin activity, Folin's lowery method

INTRODUCTION

Customer exposure to a nutritious and balanced diet has cheer scientific research in the food manufacturing to investigate and introduce natural compounds in food processing and preservation technology, to reduce the use of chemicals to inhibit microbial contamination and increase a shelf life of food products. A careful and comprehensive labour to combat the foodborne disease is a high complex challenge that involves expertise in the field of science and technology for the safety, control and regulation of food [1].

Foodborne disease has been universal problem. Despite, the use of contemporary food preservation methods, the rate of food related illness still rises and is substantial cause of death of many people, especially in countries where there is a lack of proper food safety monitoring technology. About one-third of the world population is suffering from food related ill health each year due to the intake of contaminated or intoxicated foods such as, canned food, meat, poultry, and fermented dairy products [2].

Lactic acid bacteria are generally recognized as safe (GRAS) microorganisms and they are broadly used as foods and feeds fermentation and preservatives added under well-ordered environments. Lactic acid bacteria are gram Positive, anaerobes that are nonsporulating rods or cocci and show a Negative catalase test. In the course of the fermentation of carbohydrates, they produce lactic acid as the primary end product and tolerate extremely low level of pH. *Lactobacillus* is the largest genus used in the production of variety of food like milk, yoghurt, cheese also non-dairy products such as, pickles, beer, wine, cider, chocolate and other fermented foods. These are also used to manufacture the animal feeds, e.g., silage [3].

Moreover, numerous Gram-negative bacteria like *E. coli, Salmonella, Shigella, Listeria* and *Vibrio* have been used as the test organisms to explore the antagonistic activity of newly isolated antimicrobial peptides. Although, Bacteriocin has been isolated from many strains of *Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus acidophilus,* and *Lactobacillus lactis,* it has shown the notable activity against a pathogen. Nevertheless, scientists are exploring novel strains from different food products to

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Vol 6, Issue 7, July 2021

sort out the problem of most common foodborne infections and disease [4].

Bacteriocin is a protein or complex proteins which are ribosomally synthesized compound, that is, lethal to bacteria and biologically active. Bacteriocin are divided into four group [5] [6],I) Lantibiotics;I) small hydrophobic heat-stable peptides (<13,000 Da);III) large heat-labile proteins (>30,000 Da) and IV) complex bacteriocins showing the complex molecule protein with lipid or carbohydrate.

Bacteriocin producing strain can be used as probiotics. Bacteriocin can be used as food additives, in the treatment of pathogen associated diseases and cancer therapy [7] [8]. Indeed, some infectious disease caused by pathogenic strain of Gram-positive bacteria such as *Staphylococci, micrococci*, and *Streptococci* can be prevented by bacteriocins [9]. The extensive use of classical antibiotics in treatment of human and animal diseases it leads to antibiotic resistance and has been considered a problem [10] [11] [12]. As a consequence, multiple resistant strains appeared and spread causing difficulties and the restricted use of antibiotics as growth promoters. So, the continued manufacturing of new classes of the antimicrobial agent has become of increasing importance for medicine [13] [14]. In order to control their abusive use in food products, one alternative is the application of some bacterial peptides as an antimicrobial substance in place of antibiotics of human application. Among them, bacteriocins have attracted increasing attention, since they are achieved in a nanomolar range and have no toxicity. The purpose of the current study was to achieve purification of bacteriocin synthesized by *Lactobacillus* spp. found in curd sample. This purified bacteriocin was further tasted for their antimicrobial activity and optimization of various parameters for their stability.

METHODOLOGY

Isolation of *Lactobacilli*:

Lactobacilli was isolated from the curd sample by streaking the culture on DeMannRogosa Sharp (MRS) agar plates HiMedia Laboratories. MRS medium composed of Peptone, 5gm; Tri ammonium citrate, 2 gm; $MgSO_4 \bullet 7H_2O$, 2 gm; $MgSO_4 \bullet 4H_2O$, 0.05 gm; Meat extract, 8 gm; yeast extract, 4 gm; D(+) Glucose, 20 gm; K_2HPO_4, 2 gm; Sodium acetate$\bullet 3H_2O$, 5 gm; Agar, 30 gm distilled water up to 1,000 mL. The pH of this medium was adjusted to 6.5. Then the plates were incubated anaerobically in anaerobic jar for 48h at 37°C and pure culture was examined for the morphological and colony characteristics.

Maintenance of indicator bacteria and test cultures:

The pure culture of *Lactobacillus* was maintained on MRS agar slant likewise, the susceptible organisms included both the Gram positive such as *Staphylococcus aureus* and *Bacillus subtilis* as well as Gram negative bacteria like *Pseudomonas aeruginosa* and *Escherichia coli* at 4°C and they were sub-cultured regularly.

Screening for antimicrobial activity:

The antimicrobial activity of the isolate was determined by agar well diffusion assay.

Inoculum preparation:

Culture of *Lactobacillus* was used for the production of bacteriocin. For that the colony obtain from the pure slant was inoculated into MRS broth and incubated at 37°C for 48h in static condition to obtain active culture and the cell density was measured by spectrophotometer at 600nm using sterile MRS broth as blank. The optical density of the suspension was calibrated to 1.00 O.D.

Fermentation:

Lactobacilli were grown in the MRS broth by inoculating viable active inoculum culture at the rate of 10% and incubating for 48h in a static condition at 37° C. During this period the bacteriocin was secreted in to the surrounding medium. MRS broth was composed of sodium acetate trihydrate, 5 gm; Triammonium citrate, 2 gm; Magnesium sulphateheptahydrate, 0.2 gm; Magnesiumsulphatetetrahydrate, 0.05 gm; Peptone, 10gm; Meat extract, 8 gm; Yeast extract, 4 gm; D(+) Glucose, 20 gm; Dipotassium hydrogen phosphate, 2 gm; and distilled water up to 1,000 mL and the pH was adjusted at 7 and medium was sterilized by autoclaving for 15 minute at 121°C.

Extraction and purification of Bacteriocin

Extraction of the bacteriocin:

The *Lactobacilli* was grown in MRS broth at 37°C for 48h and cell free supernatant was obtained by centrifuging at 10,000 rpm for 10 min at 4°C followed by filtration through Whatman filter paper.

Precipitation of bacteriocin:

Culture supernatant was treated with solid ammonium sulphate to 20%, 40%, 60%, 80% and 100% saturation. The mixture was then stirred for 4h at 4°C and after that centrifuged at 10,000 rpm for 30 min to precipitate the protein the inhibitory activity of the bacteriocin was checked at each stage of precipitation process.

Salting out by dialysis:

The pellet obtained after ammonium sulphate precipitation, was resuspended in 0.05M Potassium phosphate buffer pH 7. Dialysis was carried out against same buffer for 24h in dialysis bag.

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 6, Issue 7, July 2021

Purification of bacteriocin:

Bacteriocin was purified by using DEAE Sephadex A-25 column. Here, the same buffer used for mobile phase.

Estimation of total protein:

The concentration of total protein present in sample was estimated by the Folin's Lowery method.

Analytical Methods

Bacteriocin bioassay:

The antimicrobial activity was determined by agar well diffusion assay by talking 100 µL of aliquots.

Inhibition zone measurement:

The zone of inhibition surrounding the well was measured by using zone measurement scale of HiMedia.

Determination of unit activity of bacteriocin:

The quantity of bacteriocin production was calculated as Arbitrary Unit. An unit activity of the bacteriocin was defined as the activity unit (AU); 1 AU is an unit area of inhibition zone per unit volume, in this case mm^2/mL. The bacteriocin activity was calculated using following formula:

Unit Bacteriocin activity (mm^2/mL) = $\frac{Lz-Ls}{V}$

Lz = clear zone area (mm^2)

Ls = well area (mm^2)

Where, ($mm^2 = \pi r^2$)

Optimization of Bacteriocin for their Stability

Effect of enzymatic treatment on bacteriocin:

Inoculate 2 mg/mL of Proteinase-K, α-amylase, Lysozyme and Pectinase to bacteriocin and incubated at 37°C for 30 min. Take aliquots of 100 µL and perform antimicrobial activity by well diffusion method against *E. coli, P. aeruginosa, B. subtilis* and *S. aureus*.

Determination of purified bacteriocin titer:

Prepare serial dilution of bacteriocin such as 1:2, 1:4, 1:6, 1:8 of purified bacteriocin in saline solution. Take aliquots of 100 µL and perform antimicrobial activity by well diffusion method against *E. coli, P. aeruginosa, B. subtilis* and *S. aureus*.

Stability to heat:

Bacteriocin sample was expose to various temperature viz. 37°C, 50°C, 70°C, 100°C and 121°C for 30 min. Take aliquots of 100 µL and perform antimicrobial activity by well diffusion method against *E. coli, P. aeruginosa, B. subtilis* and *S. aureus*.

Stability to pH:

Adjust the pH of bacteriocin from 5 to 9 using 0.1N NaOH and 0.1N HCl. Incubate for 30 min at room temperature. Take aliquots of 100 µL and perform antimicrobial activity by well diffusion method against *E. coli, P. aeruginosa, B. subtilis* and *S. aureus*.

Effect of organic solvents on bacteriocin:

Bacteriocin mixed with acetone, chloroform, ethanol, propane n-ol at 1:1 v/v ratio and centrifuged at 10,000 rpm for 10 min at 4°C. Evaporate organic solvent. Take aliquots of 100 µL and perform antimicrobial activity by well diffusion method against *E. coli, P. aeruginosa, B. subtilis* and *S. aureus*.

Separation and Determination of molecular weight of the bacteriocin by SDS-PAGE:

The concentrated sample was electrophoresed on 10% SDS Polyacrylamide gel.

$$Mobility = \frac{Distance\ moved\ by\ protein\ (mm)}{Distance\ moved\ by\ tracking\ dye\ (mm)}$$

RESULTS AND DISCUSSION

Isolation:

The LAB (Lactic Acid Bacteria) strain was characterized by Gram's staining and was found to be Gram's positive, rod-shaped *bacillus*, and catalase test are negative. Colony appeared as milky white, dome-shaped and small with entire margins.

Image 1: LAB colony on MRS agar plate

Extraction and Purification of bacteriocin by ammonium sulphate and DEAE Sephadex A-25 Column:

Crude bacteriocin was extracted and purified by ammonium sulphate precipitation and DEAE Sephadex A-25 column. The bacteriocin band was observed in this column during purification shown in Image 2.

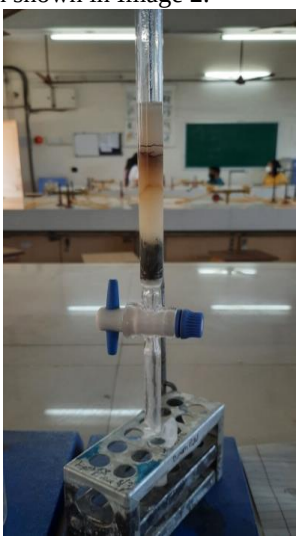

Image 2: DEAE Sephadex A-25 column

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International Journal of Science, Engineering and Management (IJSEM)
Vol 6, Issue 7, July 2021

Purified bacteriocin activity assay:

Crude bacteriocin was extracted and purified by ammonium sulphate precipitation and DEAE Sephadex A-25 column. Among all the fractions, 40% fraction was found to be displaying best activity against different test organisms. A unit activity of bacteriocin 1598.4 AU/mL was observed.

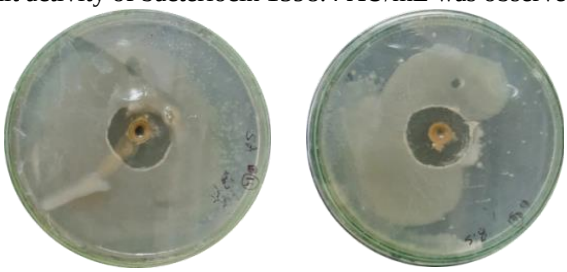


Image 3: Unit activity of pure bacteriocin against *E. coli* and *P. aeruginosa.*

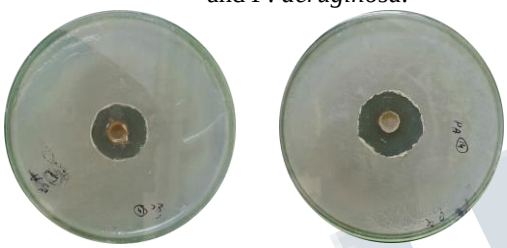
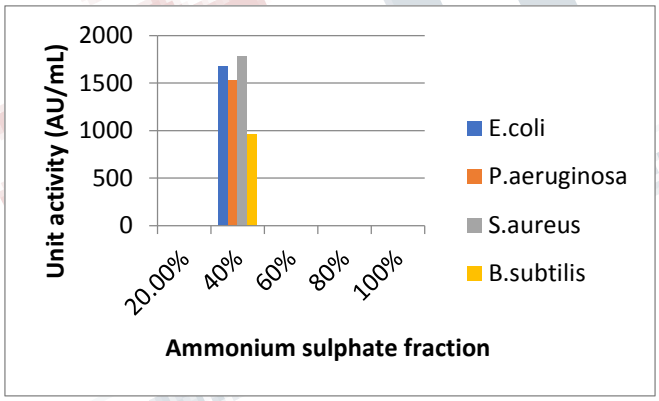


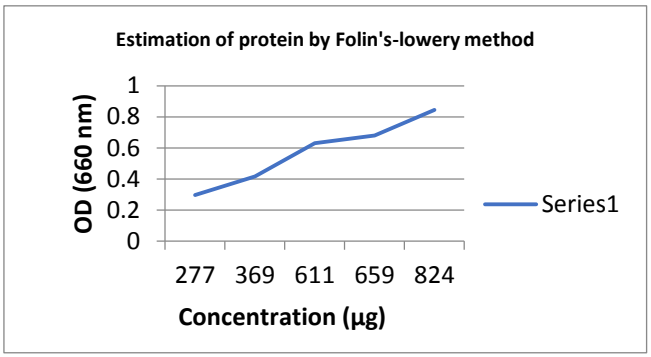
Image 4: Unit activity of pure bacteriocin against *S. aureus* and *B. subtilis.*



Graph 1: Different fraction of bacteriocin against different test microorganisms.

Estimation of total protein by Folin's Lowery method:

Purified bacteriocin was present and it can be estimated by Folin's lowery method at 660nm. The concentration 824 µg/mL was estimated. A protein reacts with the Folin-Ciocalteu's reagent (FCR) to give the blue colored complex. The color so formed is due to, the reaction of the alkaline copper with the protein and the reduction of phosphomolybdic-phosphotungstic components in the FCR by the amino acids' tyrosine and tryptophan present in the protein. The comparative data observed in graph 2.



Graph 2: Estimation of total protein by Folin's lowery method.

Optimization of Bacteriocin for their Stability

Effect of enzyme on pure bacteriocin:

There was no effect of different enzymes on purified bacteriocin activity, means it is not enzyme sensitive and the activity remained same even it treated with different enzymes [15]. The unit activity of bacteriocin 1631.44 AU/mL was observed.

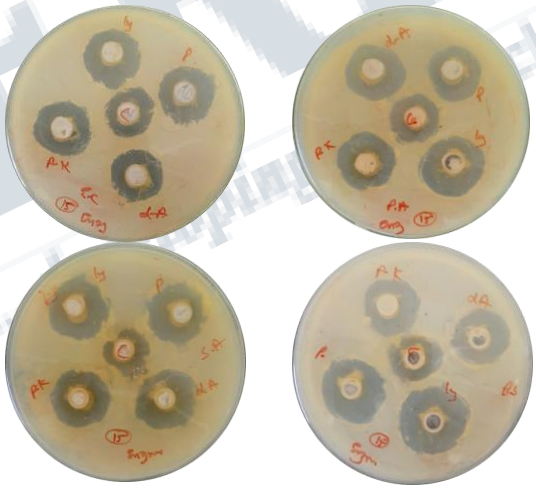


Image 5: Effect of enzyme on pure bacteriocin against *E. coli, P. aeruginosa, S. aureus and B. subtilis*

Determination of purified bacteriocin titer:

Purified bacteriocin was gave maximum activity till 1:2 dilutions. A further dilution does not give any activity which indicates that minimum inhibitory concentration was achieved at 1:2 dilutions against different test microorganisms. The unit activity of bacteriocin 2786.8 AU/mL for 1:2 dilutions was observed. The data of purified bacteriocin activity was shown in graph 3.

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 6, Issue 7, July 2021

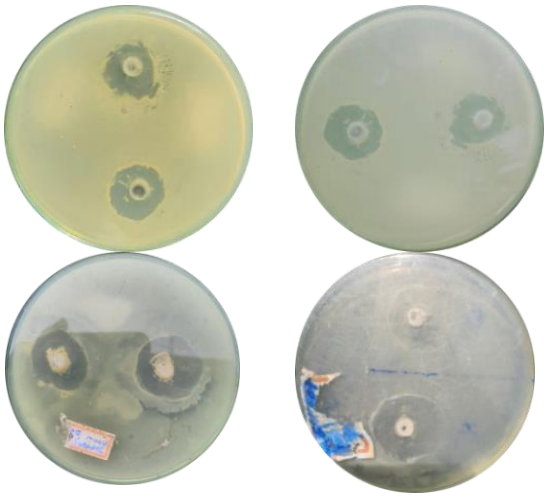
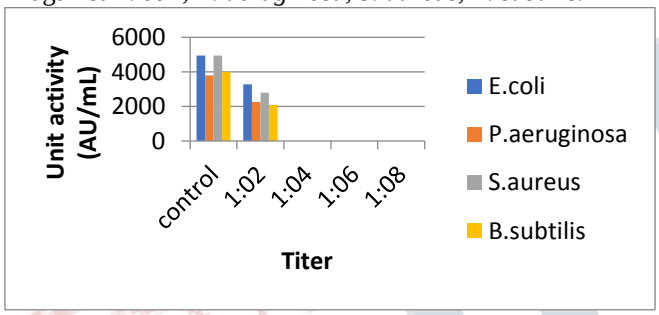


Image 6: Effect of antimicrobial titer on pure bacteriocin against *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis.*



Graph 3: Effect of antimicrobial titer on purified bacteriocin against different test microorganisms.

Stability to heat:

Studies on *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa* indicated that the pure bacteriocin was stable at higher temperature and it does not affect the activity of bacteriocin even if it can be heated at 121°C for 30 minutes [16]. For bacteriocin stability at 121°C 1631.44 AU/mL was observed.

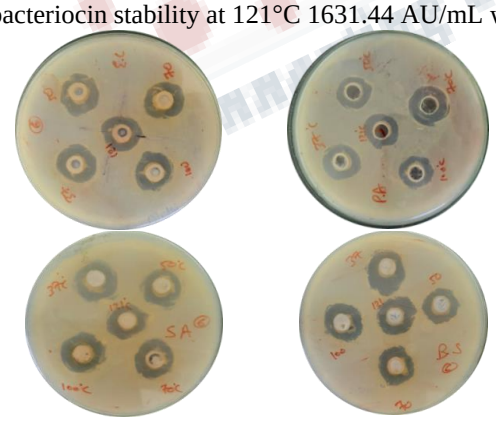
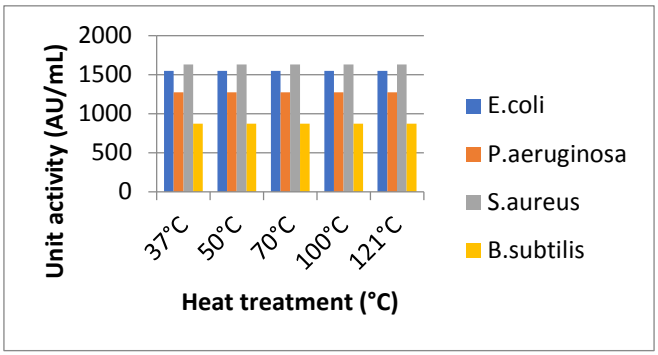


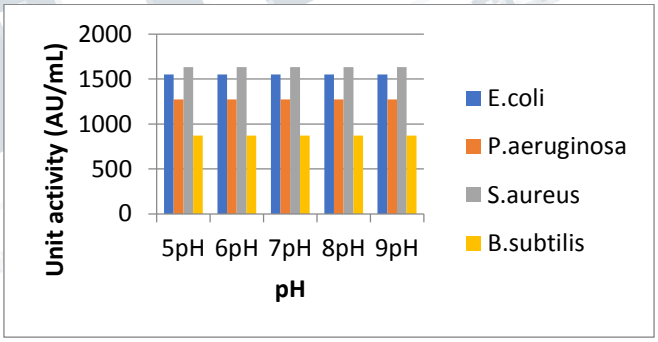
Image 7: Effect of heat on pure bacteriocin against *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis.*



Graph 4: Effect of heat on purified bacteriocin against different test microorganisms.

Stability to pH:

Studying pH stability of pure bacteriocin against *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. subtilis*, it was found that there was no effect of pH on pure bacteriocin. From that we can say that the bacteriocin was stable at pH range of from 5 to 9. The unit activity of bacteriocin 1631.44 AU/mL was observed. With respect to effect of pH, this bacteriocin have been observed to be stable over a wide pH range due to the acidic nature of its molecule [17].



Graph 5: Effect of pH on purified bacteriocin against different test microorganisms.

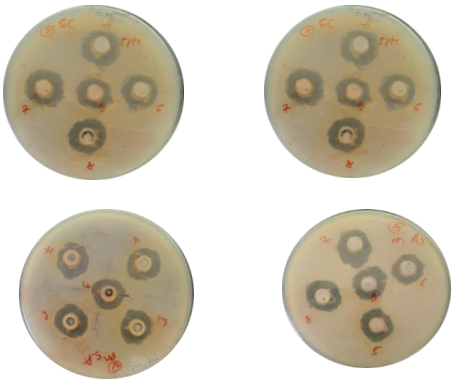


Image 8: Effect of pH on pure bacteriocin against *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis.*

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 6, Issue 7, July 2021

Effect of organic solvents on bacteriocin:

Bacteriocin treated with organic solvents shows stable in activity against different test microorganisms. So, this indicating the bacteriocin is stable to organic solvents [17]. The unit activity of bacteriocin 1631.44 was observed.

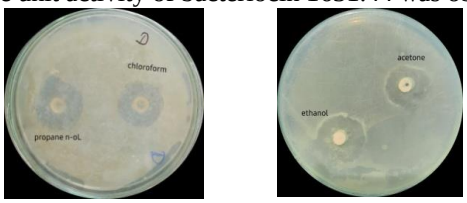


Image 9: Effect of organic solvent on pure bacteriocin against *E. coli.*

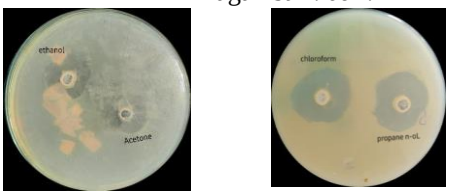


Image 10: Effect of organic solvent on pure bacteriocin against *P. aeruginosa.*

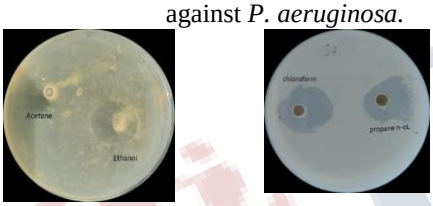


Image 11: Effect of organic solvent on pure bacteriocin against *S. aureus.*

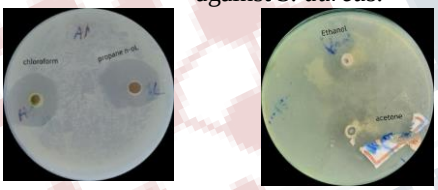
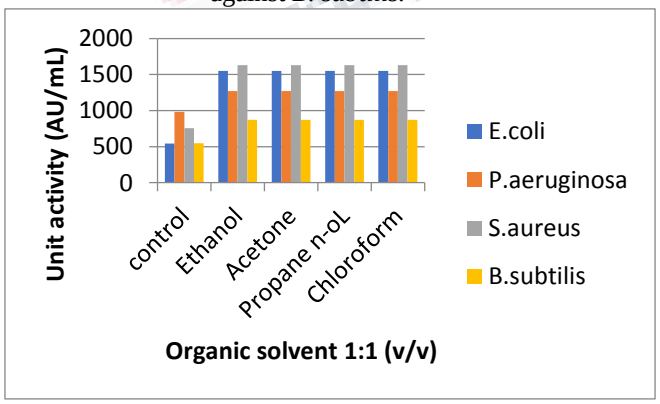


Image 12: Effect of organic solvent on pure bacteriocin against *B. subtilis.*



Graph 6: Effect of organic solvent on purified bacteriocin against different test microorganisms.

Separation and Determination of molecular weight of the bacteriocin by SDS-PAGE:

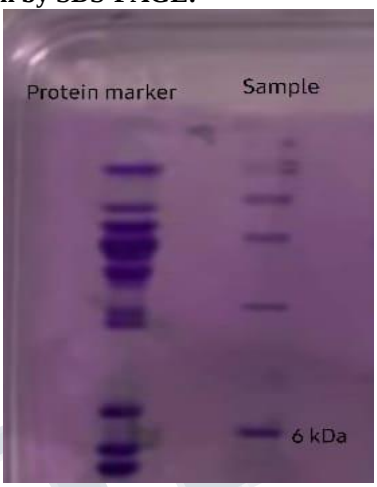


Image 13: SDS-PAGE

The SDS-PAGE analysis of purified bacteriocin was confirmed the presence of unique band with the antimicrobial activity. Its molecular weight was less than 6 kD which is comparable with its thermostability and coincides with those of others who reported that many bacteriocin of different LAB are relatively small molecule of simple structure. Therefore, this bacteriocin can be called as lanthionine due to less than 10 kD molecular weight [18].

The bacteriocin was found to be thermostable after the heat treatment of 121°C for 30 min and enzymatic treatment. Also, this SDS-PAGE shows that the bacteriocin has a low molecular weight and hence, it belongs to the class II bacteriocin which is small and thermostable.

CONCLUSION

The acceptance of probiotics by the consumers was aided greatly when this bacteriocin were marketed as natural culture, that aid in digestion and health. In the same degree, bacteriocin and bacteriocin producing medium should be attractive, especially as a consequence of consumer digest of chemical preservatives. Since bacteriocin are classified as GRAS (Generally recognized as safe) and natural products, they might have a good acceptance from purchaser who start to buy more better natural and safe food products.

The explosion of bacteriocin research has been favoured by the recognition of the mode of action that these producing bacteria may play role in the hygienic quality assurance of food and feed supplements. However, as of now only few bacteriocins are used as biopreservative. Bacteriocin produced by these microorganisms may be a good solution to the problem of resurgence of resistant strains to antibiotics. There is need to attract consumers attention to the existence of natural substance that can protect against food-borne

disease.

Overall summary, the bacteriocin extracted and purified from *Lactobacillus* spp. show vigorous antimicrobial activity against Gram positive and Gram-negative bacteria such as, *S. aureus, B. subtilis, E. coli*, and *P. aeruginosa*. Various level of activity was determined by exposing to a range of conditions of enzymes, bacteriocin titer, heat, pH, and organic solvent treatments. Based on its estimated molecular weight and the observed property of thermostability, the bacteriocin produced by *Lactobacillus* likely belongs to the Class II bacteriocin family. It is also concluded from my study that bacteriocin produced by *Lactobacilli* was effective against *S. aureus, B. subtilis, E. coli*, and *P. aeruginosa.*

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