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

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Abstract: **- From the isolated species of LAB (Lactic Acid Bacteria) shows pronounced antibacterial activity between the initial logarithmic and stationary phase. The most enormous activity in the composed medium was achieved at pH of 7, incubation temperature 37°C and incubation period of 48 h under static condition. Supplementation of nutrients demonstrated that the huge quantities of bacteriocin and could be produced by addition of glucose (2%) manipulating the dextrose (2%) and NaCl (0.5%-2.0%). Since it displayed the increase in the activity from 981.25 AU/mL to 1139.74 AU/mL. The compound produced by LAB was wholly/partially inactivated by some of the proteolytic enzymes, which confirms their proteinaceous nature. The activity of bacteriocin producing, the organism is not dependent on the glycosylation because the antimicrobial activity of bacteriocin was unaffected by the α-amylase and other enzymes. The bacteriocin shows broad range of thermo stability from 37°C to 121°C for 30 minutes. The Folin's Lowery method describes their concentration. 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 antimicrobial activity of the proteinaceous compound namely bacteriocin or BLIS (Bacteriocin like inhibitory substance) produced by Lactobacilli isolated in this work are largely identified and have been found to have potential antimicrobial activities toward closely related bacteria and undesirable harmful microorganisms. On this account, they are helpful in the field of food preservation/safety or pharmaceutical applications and healthcare.**

Keywords: Bacteriocin, LAB, Antimicrobial, Food Preservation, Folin's lowery method.

INTRODUCTION

The bacteriocins have attracted many researchers and have been studied extensively. These small proteins or peptides (typically containing less than 60 amino acids) kill or inhibit the growth of some bacteria and have narrow spectrum of bacterial growth inhibition [1] [2]. 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 [3] [4]. Indeed, some infectious disease caused by pathogenic strain of Gram-positive bacteria such as *Staphylococci*, *Micrococci*, and S*treptococci* can be prevented by bacteriocins [5]. The extensive use of classical antibiotics in treatment of human and animal diseases it leads to antibiotic resistance and has been considered a problem [6] [7] [8]. As consequences, 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 [9] [10]. 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. Bacteriocins are protein or complex proteins which are ribosomally synthesized compound, lethal to bacteria and biologically active. Bacteriocin are divided into four group [11] [12], Ⅰ) Lantibiotics; Ⅱ) 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.

Increase in consumer demand for natural preservatives has also made scientists focus on finding new natural inhibitors. Bacteriocin-producing LAB (Lactic Acid Bacteria) was used in many starter cultures to prevent pathogenic microbe from colonizing many food products. These promising characteristics of the bacteriocin and bactetiocin-producing LAB made them important not only for food preservation but also for treating certain drug-resistant pathogens [5] [13]. Biopreservation system like bacteriocinogenic lactic acid bacteria and their bacteriocin have received increasing

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attention and new approaches to control pathogenic and food spoilage microorganisms have been developed [14]. Some LAB show antagonism towards disease causing and spoilage microorganisms. Bacterial fermentation of perishable raw materials has been used for centuries to preserve the nutritive values of food and beverages over an extended shelf life. The health-conscious people may seek to avoid foods that have undergone extensive processing or which contain chemical preservatives. Among the LAB, a high diversity of bacteriocin is produced and several have been patented for their application in foods. To date, only commercially produced bacteriocins are the group of nisin, this is produced by *Lactococcus lactis* and pediocin PA-1 these, is produced by *Pediococcus acidilactici*. Minimally manufacturing refrigerated foods have been gaining consumer acceptance in the last year due to their natural appeal. However, the microbiologically safety of these foods is of concern due to the possible presence of non-proteolytic toxic strains of *Clostridium botulinum*, that is able to grow at 4°C, and the post-processing contamination with psychotrophic pathogen such as *Listeria monocytogenes*, a pathogen that have been involved in several food born out breaks worldwide and causes special concern with regard to food safety due to its psychotropic and ubiquitous characteristics. For inhibition of these bacteria there are least three ways in which bacteriocin can be incorporated into food to improve its safety [15]. I.e., using a semi or poured bacteriocin preparation as an ingredient in food, by incorporating an ingredient previously fermented with bacteriocin-producing strain, or by using direct their culture to replace all or part of a starter culture in fermented foods to produce the bacteriocins in situ. Also, bacteriocin is incorporated into food as a concentrated, though not purified, preparation made with food-grade technique. So, the bacteriocins used as a biopreservative for seafood, dairy products, and also be applied in the packaging film [16].

The purpose of the current study was to achieve the bacteriocin synthesized by *Lactobacillus* spp. found in curd sample. This bacteriocin was further tasted for their antimicrobial activity and optimization of various parameters of the growth medium for their production as well as for their stability.

METHODOLOGY

Isolation of *Lactobacilli*:

Lactobacilli were isolated from the curd sample by streaking the culture on DeMannRogosa Sharp (MRS) agar plates HiMedia Laboratories. MRS medium composed of Peptone, 5gm; Meat extract, 8 gm; yeast extract, 4 gm; D(+) Glucose, 20 gm; K_2HPO_4, 2 gm; Sodium acetate•$3H_2O$, 5 gm; Tri ammonium citrate, 2 gm; $MgSO_4$•$7H_2O$, 2 gm; $MgSO_4$•$4H_2O$, 0.05 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 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 Peptone, 10gm; Meat extract, 8 gm; Yeast extract, 4 gm; D(+) Glucose, 20 gm; Dipotassium hydrogen phosphate, 2 gm; sodium acetate trihydrate, 5 gm; Triammonium citrate, 2 gm; Magnesium sulphateheptahydrate, 0.2 gm; Magnesiumsulphatetetrahydrate, 0.05 gm; and distilled water up to 1,000 mL and the pH was adjusted at 7 and medium was sterilized by autoclaving for 15 minutes at 121°C.

Optimization of various parameter of the growth medium for the bacteriocin production

Effect of incubation time:

To determine the incubation time for the maximum bacteriocin production, the fermentation medium was inoculated with 1% v/v in sterile MRS broth and incubated at 37°C for different time interval varying from 24h, 48h, 72h, and 96h. 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 incubation temperature:

Sterile MRS broth was inoculated with 1% v/v of inoculum and incubated at different temperature such as 28°C, 37°C and 40°C. Take aliquots of 100 µL and perform antimicrobial activity by well diffusion method against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus*.

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Effect of pH:

To study the effect of different pH condition on bacteriocin production the pH of production medium was varied from 5 to 9 with the help of 1N HCl and 1N NaOH before autoclaving and then the culture was inoculated with 1% v/v of inoculum and the flask were incubated at 37°C for 48h under static condition. 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 medium composition:

Add varying amounts of Yeast extract, Beef extract, Peptone, NaCl, Dextrose and MgSO4 within MRS broth. Medium was sterilized by autoclaving at 121°C for 15 min. Inoculate 1% v/v of inoculum, each set contain of 0.5%, 1.0%, 1.5%, 2.0% w/v of these media components. The culture was withdrawn after 48h. Take aliquots of 100 µL and perform antimicrobial activity by well diffusion method against *E. coli, P. aeruginosa, B. subtilis* and *S. aureus*.

Study of bacteriocin activity

Heat resistance:

Crude bacteriocin sample were expose to various temperatures viz. 37°C, 50°C, 70°C, 100°C and 121°C for 30 min. Take aliquots of 100 µL from each of above and perform antimicrobial activity by well diffusion method against *E. coli, P. aeruginosa, B. subtilis* and *S. aureus*.

Effect of UV light:

Aliquot of 1.5 mL of crude bacteriocin was exposed to short wave UV light at a distance of 30 cm, for varying time like 10, 20, 30 and 40 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 supernatant sample from 5 to 9 pH using 0.1N NaOH and 0.1N HCl. Incubate at room temperature 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.*

Effect of surfactants:

Trion X100 and Tween 80 were added to crude sample at concentration of 0.1 mL of surfactant/mL of bacteriocin solution. SDS and Detergent powder were added at concentration of 0.01 gm of surfactant/mL of bacteriocin solution. Incubate 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.*

Effect of enzymes:

Proteinase-K, α-amylase, Lysozyme and Pectinase are inoculated in crude bacteriocin at concentration of 2mg/mL and incubate 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*.

Effect of organic solvents:

Organic solvent such as acetone, chloroform, ethanol, and propane n-ol mixed with crude bacteriocin 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.*

Bacteriocin titer:

Make a serial dilution of 1:2, 1:4, 1:6, 1:8, of 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.*

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. A unit activity of the bacteriocin was defined as activity unit (AU); 1 AU is a 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, ($mm^2 = \pi r^2$)

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.

Estimation of total protein:

By Folin's Lowery method.

RESULTS ABD DISSCUSSION

Isolation:

The LAB strain was characterized by Gram's staining after 48h of incubation (Graph 17) and was found to be Gram's positive, rod-shaped *bacillus*. And catalase test is negative. Colony appeared as milky white, dome-shaped and small with entire margins (Image 1).

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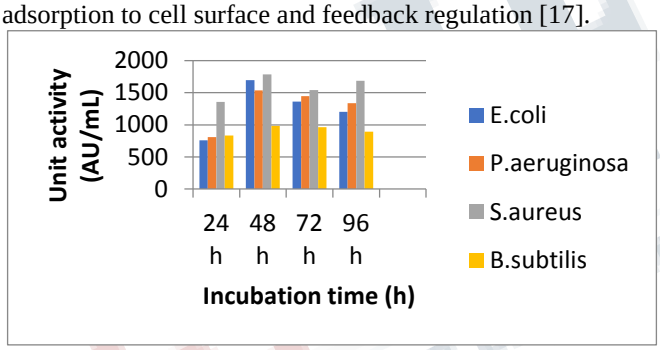
Image 1: *Lactobacilli* colony on MRS agar plate.

Optimization of various parameters for the bacteriocins production

Effect of incubation time:

The bacteriocins production was maximum at 48h of the incubation time (Image 2) (Graph 1). There was a near little bit activity observed at the 24h, was 1354.8 AU/mL, at the 72h, was 1514.65 AU/mL and at the 96h, was 1683.65 AU/mL of incubation. Although, there was increase in the number of cells. The maximum bacteriocins production occurred during early stationary phase. During extended stationary phase the activity decreased considerably. The unit activity of bacteriocin at the 48h was 1783.56 AU/mL, which is higher than other incubation time. Loss of activity has been ascribing to proteolytic degradation by endogenous extracellular protease induced during the growth phase, adsorption to cell surface and feedback regulation [17].



Graph 1: Effect of incubation time on bacteriocin production.

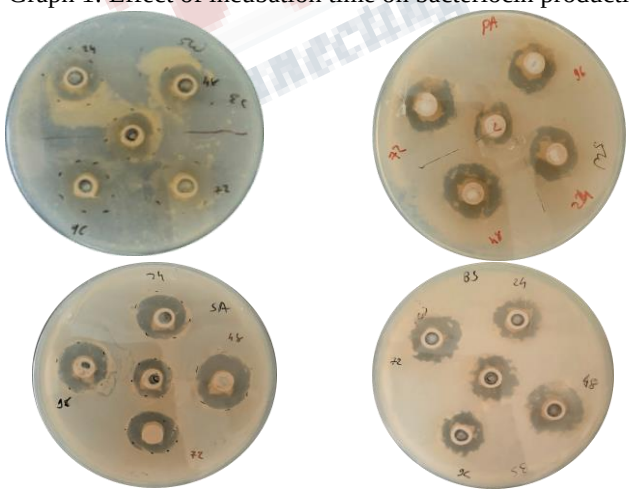
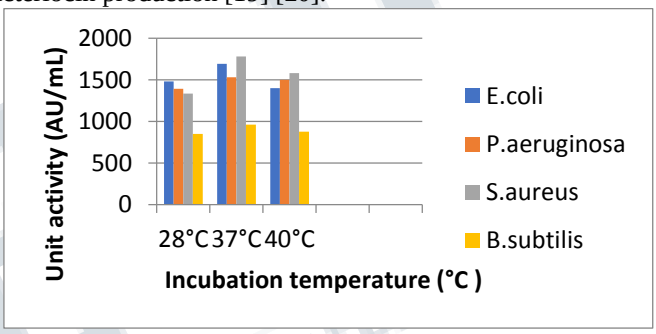


Image 2: Effect of incubation time on bacteriocin production against *E. coli, P. aeruginosa, S. aureus, B. subtilis*

Effect of incubation temperature on bacteriocin production:

The optimum temperature for the production of bacteriocin at the 37°C was 1779.98 AU/mL (Image 3), so, the bacteriocins activity at this temperature was higher than the observed at the 28°C was 1333.95 AU/mL and at the 40°C was 1577.48 AU/mL (Graph 2). According to these results we can say that, the optimum temperature for the production and the one for growth are correlated, as observed elsewhere for lactocin A, enteriocin 1146, lactocin S and nisin Z. So, the growth temperature seems to play an important role on bacteriocin production [19] [20].



Graph 2: Effect of incubation temperature on bacteriocin production against different test microorganisms.

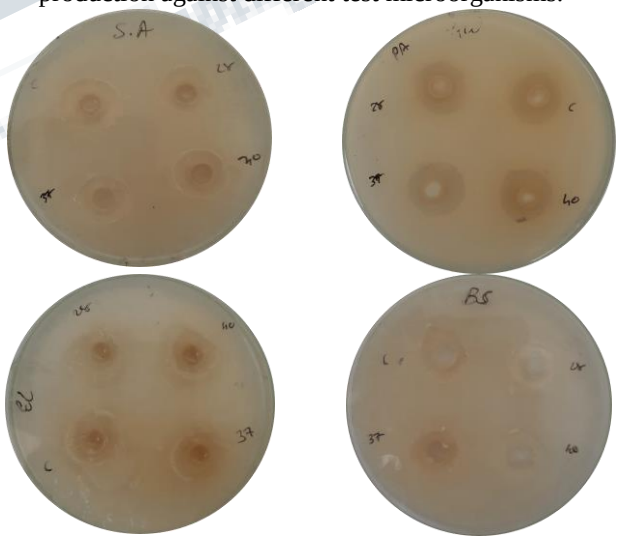


Image 3: Effect of incubation temperature on bacteriocin production against *E. coli, P. aeruginosa, S. aureus, B. subtilis*

Effect of pH:

The bacteriocin production was observed from the pH 5 to 9 but, the highest bacteriocin was observed at the 7 pH was 1773.11 AU/mL (Graph 3). However, there is considerable inhibition between this pH. It was reported that the optimal

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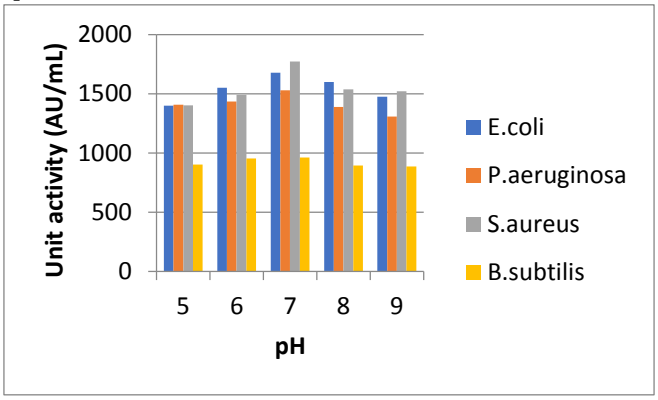
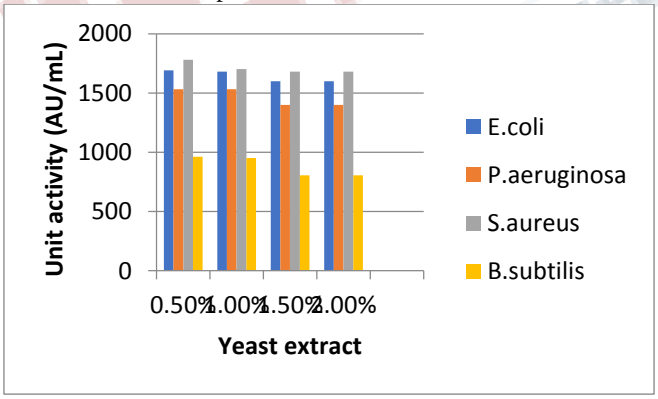
bacteriocin production by L. lactis spp. occurred between 6 to 7 pH in MRS and M17 broth at 30°C. As bacteriocin production is linked to cell growth, it also depends on factors that affect the cell growth such as pH and temperature [19] [20].



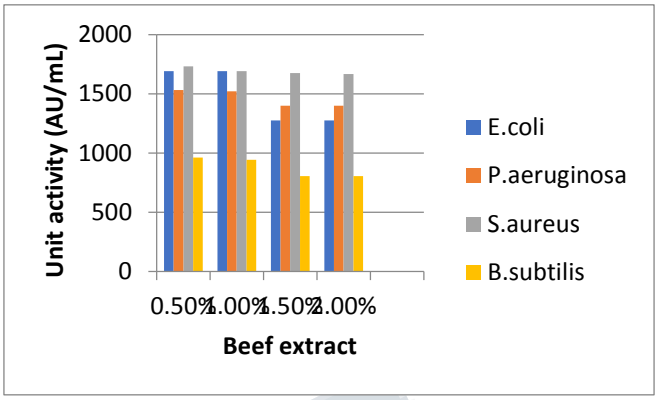
Graph 3: Effect of pH on bacteriocin production against different test microorganisms.

Effect of medium composition:

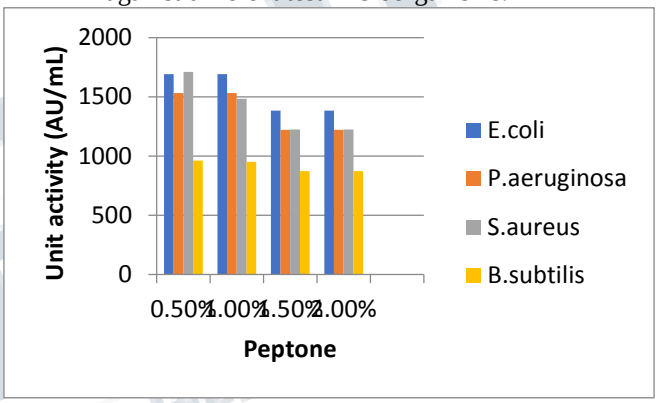
A requirement of yeast extract, beef extract, Peptone and NaCl was very less only 0.5% i.e., 0.5% of these components was enough to boosting bacteriocin production [18]. At this amount the unit activity was 1779.31 AU/mL for yeast extract, 1731.6 AU/mL for Beef extract, 1708.93 AU/mL for Peptone and 1703.2 AU/mL for NaCl. On the other hand, Dextrose (1.5%) and MgSO4 (2.0%) speed up the bacteriocin production, at this amount the unit activity was 1783.61 AU/mL for MgSO4 (2.0%), for Dextrose (1.5) was 1703.17 AU/mL and at a particular level it reaches at plateau i.e., no further increase in the production of bacteriocin.



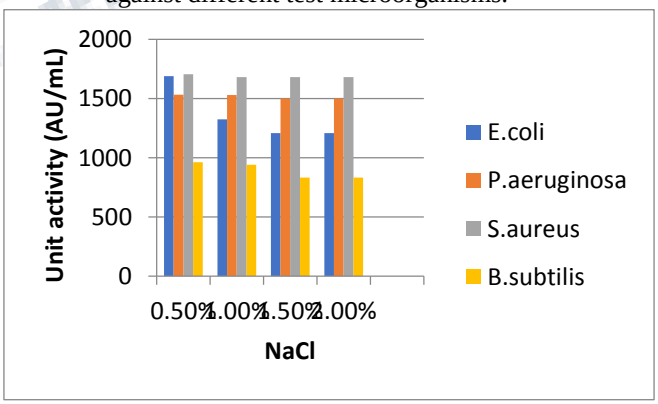
Graph 4: Effect of Yeast extract on bacteriocin production against different test microorganisms.



Graph 5: Effect of Beef extract on bacteriocin production against different test microorganisms.



Graph 6: Effect of Peptone on bacteriocin production against different test microorganisms.



Graph 7: Effect of NaCl on bacteriocin production against different test microorganisms.

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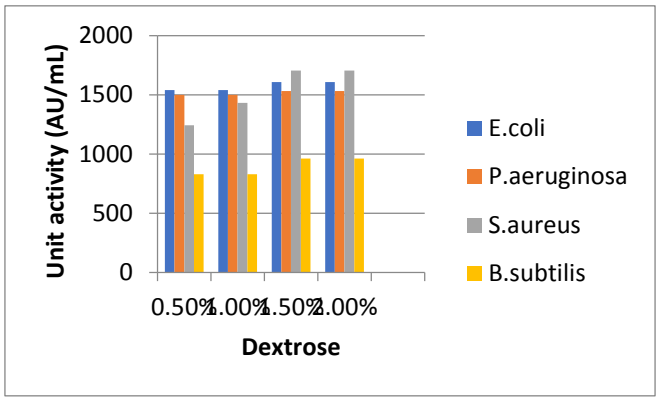
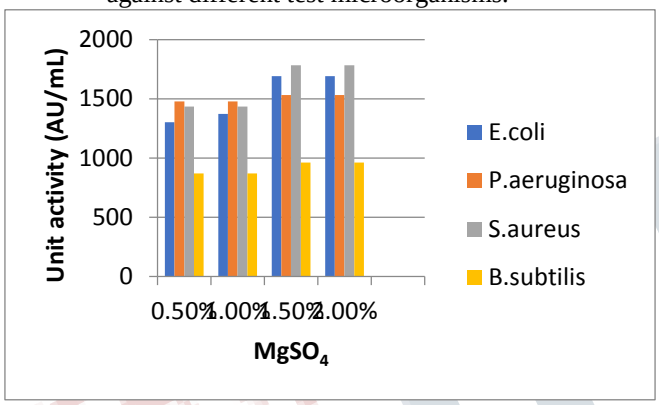


Graph 8: Effect of Dextrose on bacteriocin production against different test microorganisms.



Graph 9: Effect of $MgSO_4$ on bacteriocin production against different test microorganisms.

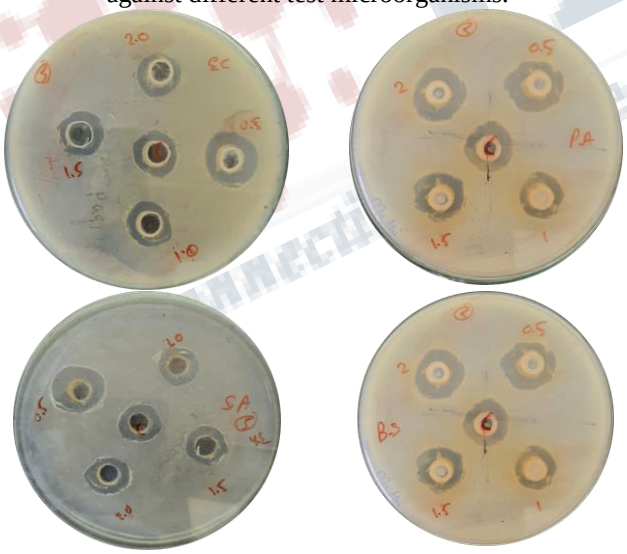
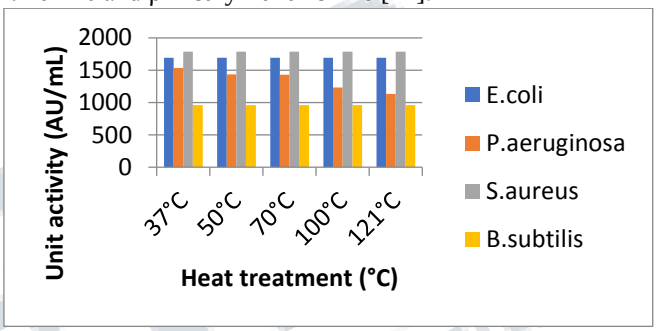


Image 4: Effect of Yeast extract on bacteriocin production against *E. coli, P. aeruginosa, S. aureus, B. subtilis* Study of bacteriocin activity.

Study of bacteriocin activity

Heat resistance:

Studies on *S. aureus, B. subtilis*, and *E. coli* indicated that the bacteriocin production 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, at this temperature the unit activity was 1783.65 AU/mL. But in cases of *P. aeruginosa* bacteriocin activity was decrease with increasing in temperature yet it displayed activity at 121°C for 30 minutes was 1132.6 AU/mL. Activity has been observed at 121 °C due to the presence of thermostable amino acids like lanthionine and β-methyl-lanthionine [21].



Graph 10: Effect of heat on bacteriocin activity against different test microorganisms.

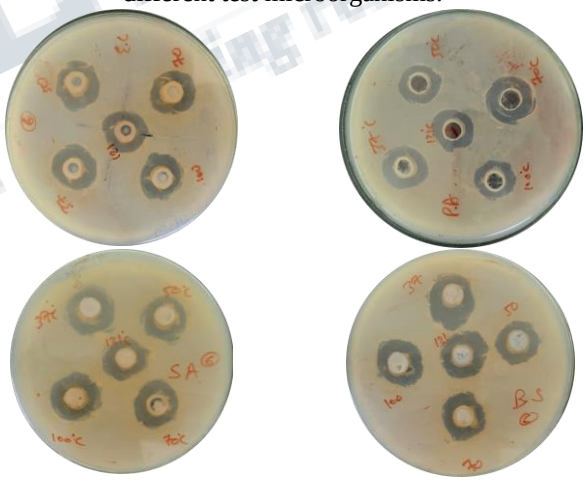


Image 5: Effect of heat on bacteriocin activity against *E. coli, P. aeruginosa, S. aureus, B. subtilis.*

Effect of UV light on bacteriocin activity:

In the case of studies with *E. coli, P. aeruginosa, B. subtilis, S. aureus*, it was found that on treating bacteriocin with UV at different time of exposure, there is no effect on bacteriocin. At this time the unit activity was 1782.72 AU/mL. Bacteriocin activity was found to be not increase or decrease by UV exposure time it means it is stable.

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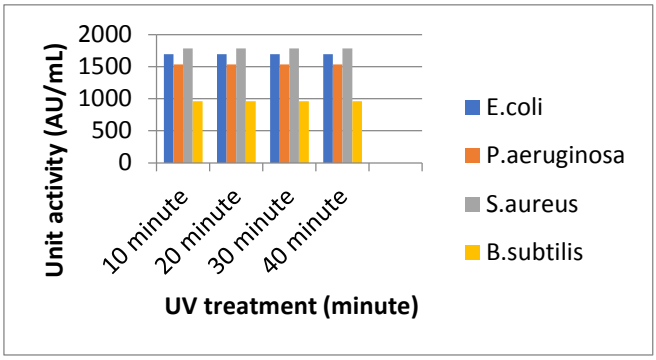
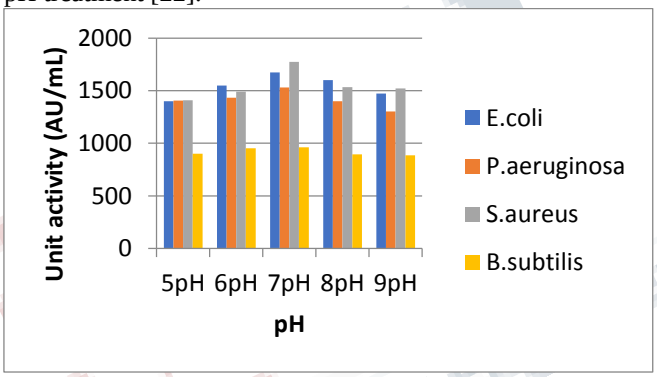


Graph 11: Effect of UV on bacteriocin activity against different test microorganisms.

Stability of bacteriocin to pH:

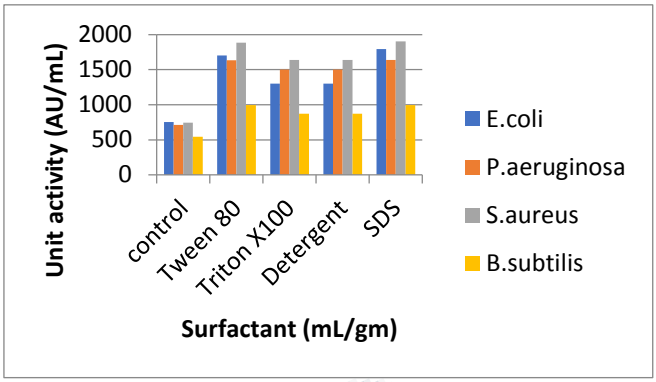
Studying pH stability of bacteriocin against *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis*, it was found that at the pH 7 the best activity was found that was 1773.11 AU/mL. At pH 5 the unit activity was 1407.49 AU/mL, for the pH 6 was found to be 1451.17 AU/mL, at the pH 8 the unity activity was 1533.39 AU/mL and 1522.1 AU/mL for pH 9was found. Bacteriocin activity decrease on further increase or decrease in pH treatment [22].



Graph 12: Effect of pH on bacteriocin activity against different test microorganisms.

Effect of surfactant on bacteriocin activity:

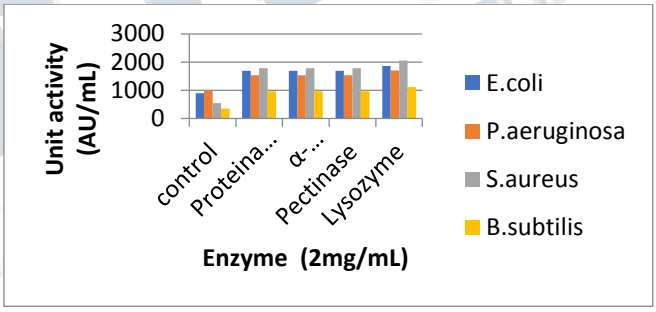
As the treatment of bacteriocin with surfactants was carried out and it shown positive change in bacteriocin activity and maximum activity was found with Tween 80 was 1883.12 AU/mL and for SDS treatment the unit activity was 1899.97 AU/mL. This increase in activity of bacteriocin may be because of the effect of the surfactants themselves [22]. For Triton X100 and Detergent the unit activity 1639.46 AU/mL was found and responsible for diminishing activity of bacteriocin.



Graph 13: Effect of surfactant on bacteriocin activity against different test microorganisms.

Effect of enzyme on bacteriocin activity:

There was no effect of enzyme such as Proteinase K, Pectinase, α-Amylase on bacteriocin activity the unit activity for these was 1783.65 AU/mL in cases of above test organisms. But on the treating bacteriocin with Lysozyme it increases the activity and that was 2048.1 AU/mL which might be because of lysis of bacterial cell wall by lysozyme itself [13].



Graph 14: Effect of enzyme on bacteriocin activity against different test microorganisms.

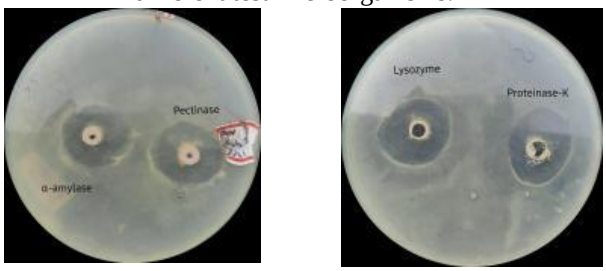


Image 6: Effect of enzyme on bacteriocin activity against *E. coli.*

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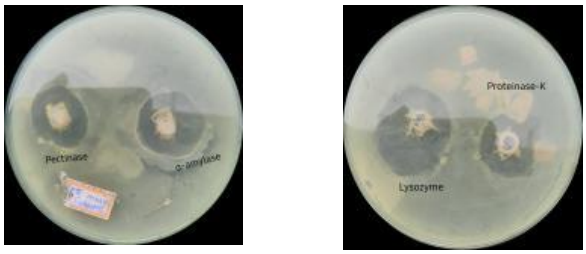


Image 7: Effect of enzyme on bacteriocin activity against *P. aeruginosa.*

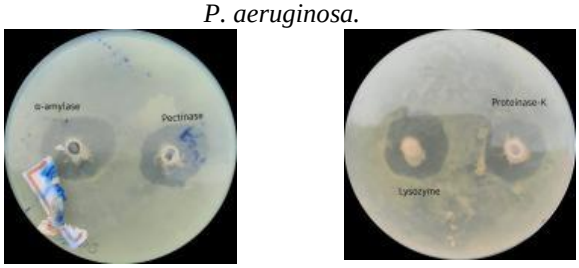


Image 8: Effect of enzyme on bacteriocin activity against *S. aureus.*

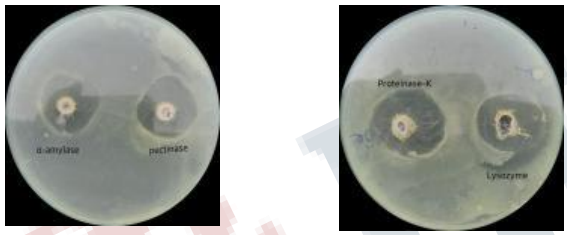
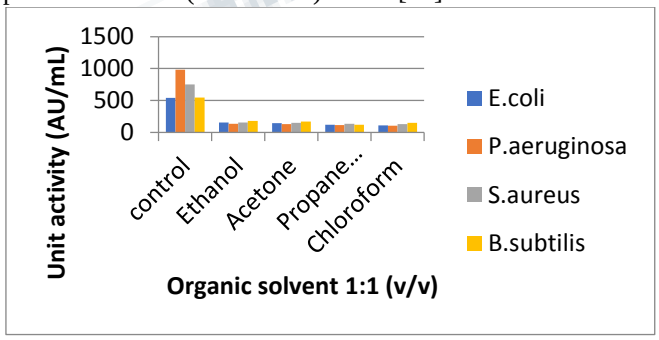


Image 9: Effect of enzyme on bacteriocin activity against *B. subtilis.*

Effect of organic solvents on bacteriocin activity:

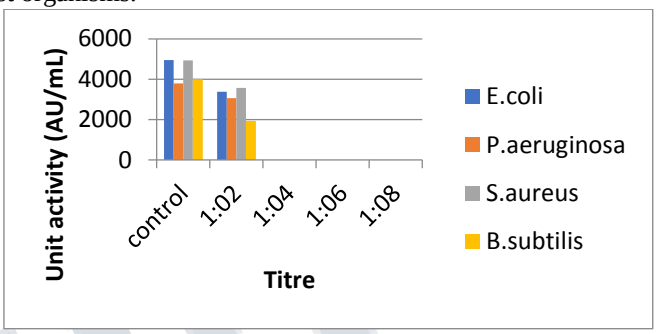
Bacteriocin treated with organic solvents shows decrease in inhibitory activity, which was 157.39 AU/mL for ethanol, 150.21 AU/mL for acetone, 137.7 AU/mL for propane n-oL and for chloroform the unit activity 131.4 AU/mL was found, that indicating the bacteriocin sensitive to organic solvents. Bacteriocins are proteinaceous in nature and this property has been utilized to ascertain whether the antimicrobial substance is protein in nature (bacteriocin) or not [22].



Graph 15: Effect of organic solvents on bacteriocin activity against different test microorganisms.

Determination of bacteriocin titer:

Bacteriocin activity was detected at 1:2 dilutions, for this titer the unit activity 3567.3 AU/mL was found, and there is no further activity detected as the dilution increase. This may be because of minimum inhibitory concentration had already present in bacteriocin. This was carried out against different test organisms.



Graph 16: Effect of antimicrobial titer on bacteriocin activity against different test microorganisms.

Image 10: Effect of antimicrobial titer on bacteriocin activity against *E. coli, P. aeruginosa, S. aureus, B. subtilis.*

Estimation of total protein by Folin's Lowery method:

Purified bacteriocin is present at a concentration of 824 µg/mL and it can be estimated by Folin's lowery method at 660nm. Protein reacts with the Folin-Ciocalteu's reagent (FCR) to give a 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 & tryptophan present in the protein. The intensity of the blue color is measured calorimetrically at 660 nm.

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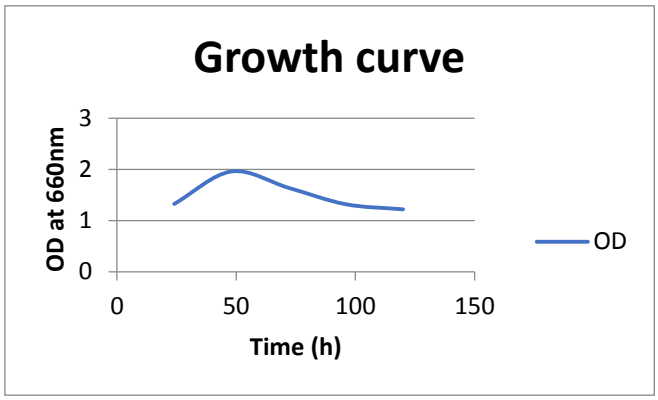

Graph: 17 The Growth Curve of *Lactobacillus* Spp.

CONCLUSION

The explosion of bacteriocin research has been favoured by the recognition of the role 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. LAB was recognized as GRAS, and 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. 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 like manner, bacteriocin, BLIS 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 order more better natural and safe food products.

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