IJNRD.ORG ISSN : 2456-4184
INTERNATIONAL JOURNAL OF NOVEL RESEARCH AND DEVELOPMENT (IJNRD) | IJNRD.ORG
An International Open Access, Peer-reviewed, Refereed Journal

PURIFICATION AND PARTIAL CHARACTERIZATION OF ANTI-MICROBIAL PEPTIDES PRODUCED BY *Bacillus licheniformis* ISOLATED FROM CURD

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ABSTRACT: *Bacillus licheniformis*, a potential saprophytic, Gram positive, endospore forming bacteria, is considered to be a species that yields a broad range of antimicrobial compounds. Natural antimicrobials are an alternative to the chemical and artificially synthesized substances which poses a threat to the health and well-being of living things. In the food industry, bacteriocins and other antimicrobial compounds derived from *B. licheniformis* are regarded as bio preservatives in preservation of food items. Potential bacterial species that produces these compounds can be screened from various sources like fermented foods, soil, water, plants, etc. The current study focuses on the purification of antimicrobial substances produced by bacterial species isolated from curd. The antimicrobial substances can be isolated from the cell free supernatant obtained by centrifugation of the bacterial culture broth. The bacterial species that produces these compounds can be identified by 16 SrRNA sequencing after carrying out the anti-bacterial activity testing. Further purification of the antimicrobial substances can be carried out by membrane filtration, ammonium sulfate precipitation, gel filtration chromatography, etc. The confirmation of protein in the purified fraction can be identified and confirmed by Lowry's method of protein estimation and SDS PAGE. The inhibition of growth of *Escherichia coli* and *Listeria monocytogenes* showed that the purified proteins from *Bacillus licheniformis* can serve as natural food preservatives after safety testing. It may also be employed as biopreservatives in cosmetics and pharmaceuticals and used to control plant pathogens in agriculture.

KEYWORDS : *Bacillus licheniformis*, antimicrobial, bio-preservative, bacteriocin, gel filtration chromatography

INTRODUCTION

Among the members of the *Bacillus* genus, *Bacillus licheniformis* is the species that yields a broad range of antibiotic compounds. The capacity of *B. licheniformis* to generate a significant number of compounds with antibacterial, antioxidant, and immunomodulatory properties is linked to its efficacy as a probiotic (Ramirez-Olea *et al.,* 2022). In the food sector, bacteriocins derived from *B. licheniformis* are regarded as bio-preservatives in food preservation (Luca *et al.,* 2002). When it comes to combating pathogens that have developed resistance to antibiotics, bacteriocins, a class of antimicrobial peptides, offer a viable substitute to traditional antibiotics (Magashi *et al.,* 2019).

Bacillus licheniformis is a saprophytic, gram-positive, endospore-forming bacteria that is found in soil and plants (Veith *et al.,* 2004). According to a taxonomical approaches, it was found to be closely linked to *Bacillus subtilis* (Lapidus *et al.,* 2002). Because of the potential of its extracellular products for commercial use, *B. licheniformis* is an economically beneficial species (Kovacs., 2009). It is employed industrially to produce aminopeptidase, enzymes, biochemicals, and antibiotics.

Bacteriocins are compounds that function against other bacterial strains or closely related species. They are represented by an amino acid sequence (peptides or proteins). They exhibit effects that are both bacteriostatic and bactericidal. Bacteria ribosomally synthesise natural antibacterial peptides called bacteriocins (Yang *et al.,* 2014). The stability of the antimicrobial peptides generated by *Bacillus* species varies with respect to temperature and pH, as does their resistance to enzyme activity (Mercado *et al.,* 2022). Although *B. licheniformis* produces a variety of bacteriocins with molecular weights ranging from 1.4 to 55 kDa, the

expression of a given antimicrobial agent can vary depending on the growth phase, the environment, and the strain of bacteria in question (Luca *et al.*, 2002).

Special non-ribosomal multimodular peptide synthetases, primarily present in bacteria and fungi, sequentially condense amino acids to produce non-ribosomal peptides. (Finking and Marahiel., 2004). Numerous well-known chemicals, including immunosuppressants (cyclosporine), antitumor agents (bleomycin), and antibacterial medications (penicillin and vancomycin), are examples of such peptides (Süssmuth *et al.*, 2017). Bacitracin is a non-ribosomally synthesised dodecapeptide antibiotic, with a molecular weight of approximately 1.42 kDa, that is generated by specific strains of *B. subtilis* and *B. licheniformis* (Jin *et al.*, 2020).

Exopolysaccharides, or EPSs, are high molecular weight polymers made up of repeating units of sugar moieties that are linked to a carrier lipid (Angelin and Kavitha., 2020). EPSs have shown antibacterial efficacy against Gram-positive and Gram-negative bacterial infections in certain instances. It was discovered that they had antagonistic action towards fungus, viruses, and bacteria. Additionally, EPSs prevent pathogenic bacteria from colonising different surfaces and from forming biofilms (Abdalla *et al.*, 2021). *B. licheniformis* is capable of producing EPSs with a range of biological activities, including antioxidant and antibacterial effects. One typical example is levan (fructan), which has antibacterial and antioxidant properties against *Pseudomonas aeruginosa*, *E. coli*, and *Staphylococcus aureus* (Hertadi *et al.*, 2021).

MATERIALS AND METHODS

1. ISOLATION AND PURE CULTURE PREPARATION OF MICROORGANISMS PRESENT IN CURD.

Curd is a rich source of probiotic lactic acid bacteria and other beneficial *Bacillus* species. Homemade curd was used as inoculum in preparing curd from raw milk. Serial dilution of curd sample was carried out in 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and10^{-5} dilutions**.** The diluted samples were plated onto MRS (deMan Rogosa Sharpe) agar and incubated at 30°C for 48 hours (Timothy *et al.*, 2014).

The different colonies observed on the mother plate were plated onto separate MRS agar plates. The plates were sub cultured into MRS broth and nutrient broth at an interval of 2 weeks (Timothy *et al.*, 2014).

2. EXTRACTION OF CELL FREE SUPERNATANT FROM THE BACTERIAL CULTURES

The pure cultures were inoculated into MRS broth separately and incubated for 72 hours at 30°C. The cell free supernatant of the bacterial culture medium was extracted by double centrifugation at 3000 rpm for 15 minutes. The settled bacterial cells were removed and the supernatant was collected (Timothy *et al.*, 2014).

3. TESTING OF ANTI-BACTERIAL ACTIVITY OF CELL FREE SUPERNATANTS AGAINST *Listeria monocytogenes.*

The anti-microbial activity of the culture supernatants of the isolated microorganisms were tested against *Listeria monocytogenes* by agar well diffusion method on nutrient agar by taking 50µl and 100µl concentrations of the cell free supernatant (Timothy *et al.*,2014).

4. PERFORM THE BIOCHEMICAL CHARACTERIZATION OF THE BACTERIAL ISOLATE

4.1) Gram's staining

A loop full of bacterial culture was taken on a glass slide and heat fixed. 2 drops of crystal violet solution was added and allowed to flood the slide for 1 minute. The stain was washed away with water. 2 drops of Gram's iodine solution was added and the slide was flooded for 1minute. A decolourizing agent (ethanol) was applied to the slides and then washed with distilled water. 2 drops of saffranin solution was added and allowed to stand for 1 minute followed by washing with distilled water. The slide was allowed to dry and then observed under a microscope (Becerra *et al.*, 2016).

4.2) Catalase test

The slide drop method was performed. A loopful of bacterial culture was taken on a clean glass slide placed in a petri dish and 1 drop of hydrogen peroxide was added with a dropper and observed for the formation of bubbles (Reiner and Karen, 2010).

4.3) Citrate utilization test

A loopful of bacterial culture was streaked onto Simmon's citrate agar and incubated at 35°C for 48 hours under aerobic conditions (MacWilliams *et al.*, 2009).

4.4) Indole test

4 ml of sterilized tryptophan broth was taken in a test tube and a loopful of bacterial culture was inoculated. The tube was incubated at 35°C for 24 to 48 hrs followed by addition of 5 drops of Kovac's reagent to the broth culture (MacWilliams *et al.*, 2012).

4.5) Methyl red test

Test tube containing 5 ml MR-VP medium (Glucose Phosphate Broth) was inoculated with a pure culture of the organism. The tube was inoculated at 35°C for 48 hours under aerobic conditions. 5 to 6 drops of methyl red reagent was added to 2.5 ml of the broth (McDevitt *et al.*, 2009).

4.6) Voges proskauer test

Test tube containing 5 ml MR-VP medium (Glucose Phosphate Broth) was inoculated with the bacterial culture and the tube was incubated aerobically at 35°C. Following incubation, 2.5 ml of the broth was taken and 12 drops of Barritt's reagent A (5% α-naphthol solution) was added and mixed properly by shaking. Then 4 drops of Barritt's Reagent B (40% KOH solution) was added and mixed properly by shaking and kept for 30 minutes (McDevitt *et al.*, 2009).

5. IDENTIFICATION OF THE BACTERIAL ISOLATE BY 16SrRNA SEQUENCING

Genomic DNA Isolation from Bacteria

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions.

Agarose Gel Electrophoresis for DNA Quality and Quantity check

The quality of the DNA isolated was checked using agarose gel electrophoresis. The gels were visualized in a UV

transilluminator (Genei) and the image was captured under UV light using Gel documentation system.

PCR Analysis

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels and the gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

ExoSAP-IT Treatment

ExoSAP-IT (USB) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product is mixed with 0.5µl of ExoSAP-IT and incubated at 37ºC for 15 minutes followed by enzyme inactivation at 85ºC for 5 minutes.

Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufactures protocol.

Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond et al., 2010).

6. CONFIRMATION OF AMYLASE PRODUCTION BY THE BACTERIAL SPECIES BY STARCH HYDROLYSIS TEST

Starch agar medium was prepared and streaked with the bacterial species and incubated at 37 ºC for 24 hours followed which utilization of starch by the organism was studied by flooding the plate with iodine solution and observing for the formation of a clear zone around the bacteria (Evans *et al.,* 2004).

7. CONFIRMATION OF PROTEINS IN THE CELL FREE SUPERNATANT OBTAINED FROM *Bacillus licheniformis* BY BIURET METHOD

Presence of protein in the cell free supernatant was identified by noting the colour change during the addition of biuret reagent (Pratt *et al.,* 2011).

8. PURIFICATION OF PROTEINS BY GEL FILTRATION CHROMATOGRAPHY

Gel filtration chromatography was carried out by using silica gel G -75 as the gel and phosphate buffer as the eluent. The flow rate was adjusted to 1ml per minute. 50 ml fractions of phosphate buffer with pH 6, 6.5, 7, 7.5 and 8 were taken for elution of 5 ml sample for each pH. (Ó'Fágáin *et al.,* 2011).

9. IDENTIFICATION OF PROTEIN CONCENTRATION BY BIURET METHOD

1ml of Biuret reagent was added to 1 ml of each fractions collected from each pH (Pratt *et al.,* 2011).

10. TESTING OF ANTI BACTERIAL ACTIVITY AGAINST *Listeria monocytogenes* and *Escherichia coli.*

The fractions identified with the presence of more concentration of protein were selected and assayed for anti-bacterial activity against *Listeria monocytogenes* and *Escherichia coli* using agar well diffusion method (Timothy *et al*., 2014).

11. ESTIMATION OF PROTEIN BY LOWRY METHOD

The concentration of protein present in the active fractions tested for anti-microbial activity was selected and Lowry's method of protein estimation was carried out by taking the absorbance at 660nm followed by plotting of graph to determine the protein concentration (Noble *et al.,* 2009).

12. SEPARATION AND CONFIRMATION OF PROTEIN USING SDS PAGE

Acrylamide and Bis Acrylamide gel polymerization was allowed to complete for about 15-30 minutes using APS and TEMED. In the meantime, the sample was prepared. The test was mixed with the sample buffer in the ratio of 3:1 and it was boiled for 1-2 minutes. After polymerization was over, the comb was carefully removed, the wells were rinsed with tank buffer and the bottom spacer was removed. The gel plate was clamped and it was tightened with the vertical gel electrophoresis apparatus. Using a micropipette, the sample was loaded to each well. Slowly the top chamber was filled with tank buffer and tank buffer was added to the top and bottom reservoir. The tank was connected to the power supply and the power was turned on. The power supply was set to constant current mode (100Mv).The system was continued till the marker dye reaches the bottom of the gel, it was turned off and the power supply was disconnected. The gel from the glass plate was carefully taken and transferred to a plastic tray. The CBB G-250 staining solution was poured into the gel tray and it was placed on the rocker for overnight. After overnight incubation the excess amount of stain was removed by using destaining solution. The gel was transferred onto the transilluminator and the protein bands were visualized.

RESULTS AND DISCUSSION

1. ISOLATION AND PURE CULTURE PREPARATION OF MICROORGANISMS PRESENT IN CURD

After the incubation period of 48 hours, different colonies were found growing on the MRS agar plates. Round solid colonies and round cloudy colonies were observed by analyzing the colony morphology.

Pure culture was prepared by streaking the individual colonies on MRS agar which can be seen grown on the agar plates after incubation. Subsequent sub culturing was also done in MRS broth and nutrient broth to obtain liquid culture. The plates and the liquid broth were stored at 4 °C for further study. According to a study, regular subculturing was done at an interval of 10 days

(Abhinav, and Nimisha Dutta, 2016).

2. EXTRACTION OF CELL FREE SUPERNATANT FROM THE BACTERIAL CULTURES

The bacterial cells were sedimented and the cell free supernatant was obtained. Studies showed that a pure supernanatnt was obtained by double centrifugation carried out at 3000 rpm for 15 minutes. Another study reported a membrane filtration technique after centrifugation by using 0.45 μm pore size filters (Liu, Huaying, *et al.*, 2023).

3. TESTING OF ANTI-BACTERIAL ACTIVITY OF THE CELL FREE SUPERNATANTS AGAINST *Listeria monocytogenes.*

After incubation, the Cell free supernatant obtained from the bacterial species was found to exhibit a low antimicrobial activity against *Listeria monocytogenes* as only a negligible zone of inhibition of approximately 4mm radius was obtained. Studies reported higher zones of inhibition for certain isolates and lower zones of inhibition for others depending on the bacterial species employed in the study (Timothy *et al*., 2014).

4. BIOCHEMICAL CHARACTERIZATION OF THE BACTERIAL ISOLATE 2

4.1. Gram's staining

The bacterial smear stained purple in colour after observing at 40x resolution under the microscope which indicated that the bacterial isolate was Gram positive and rod shaped in nature.

4.2. Catalase test

Formation of air bubbles were observed on the glass slide after addition of hydrogen peroxide which indicated that the organism was capable of producing catalase enzyme and the consequent decomposition of hydrogen peroxide into oxygen and water. Therefore the bacterial isolate was found to be catalase positive.

4.3. Citrate utilization test

No colour change was observed which indicated that the organism cannot utilize citrate as energy source and was incapable of producing an enzyme called citrate permease which converts citrate into pyruvate. So the bacterial isolate was found to be citrate negative.

4.4. Indole test

No colour change after the addition of the reagent and the absence of ring indicates that the organism was incapable of decomposing the amino acid tryptophan to indole. So the bacterial isolate was found to be indole negative.

4.5. Methyl red test

The absence of bright red colour after the addition of reagents indicated that the organism was incapable of utilizing glucose to produce a stable acid. So the bacterial isolate was found to be methyl red negative.

4.6. Voges proskauer test

The absence of pink-red colour over the surface of the medium indicated that the organism was incapable of metabolizing pyruvate into a neutral intermediate product acetoin. So the bacterial isolate was found to be negative for Voges Proskauer test.

5. 16SrRNA SEQUENCING OF THE BACTERIAL ISOLATE 2

16SrRNA sequencing was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufactures protocol. The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond et al., 2010).

The FASTA sequence was analyzed by carrying out NCBI nucleotide BLAST and a phylogenetic tree was constructed using MEGA 11 software.

From the data obtained, the isolated bacterial species was inferred to be *Bacillus licheniformis*.

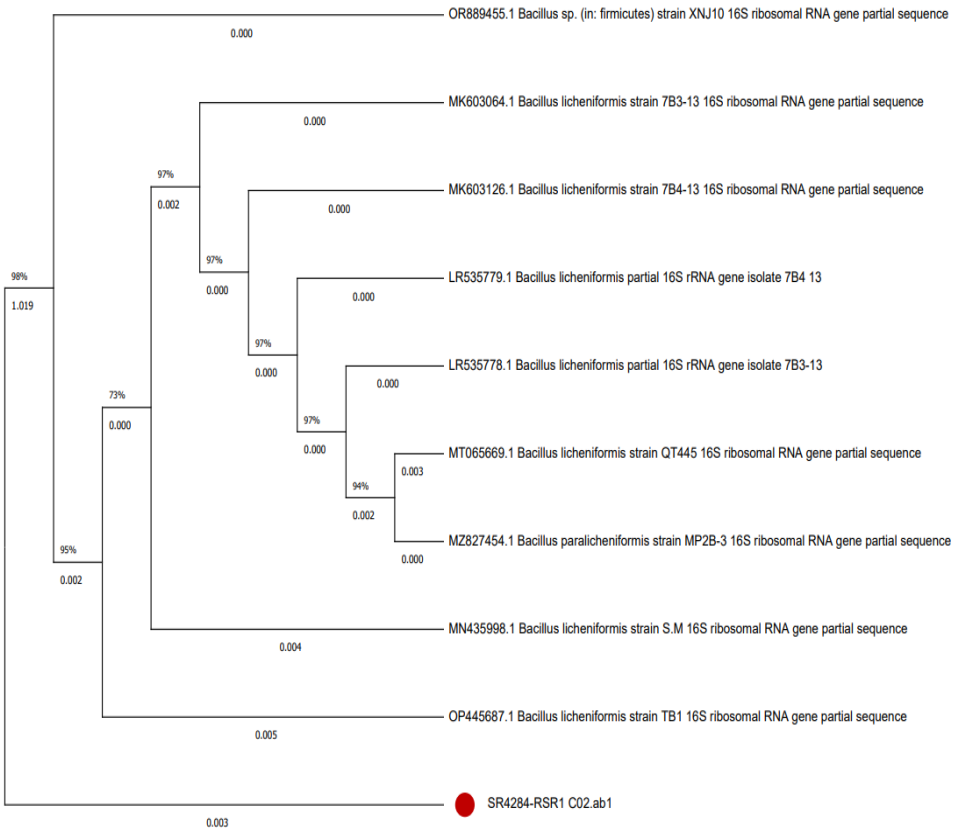


Fig. phylogenetic tree

6. CONFIRMATION OF AMYLASE PRODUCTION BY THE BACTERIAL SPECIES USING STARCH HYDROLYSIS TEST

After the incubation period, a clear zone around the bacteria indicated that the bacterial isolate was capable of producing alpha amylase enzyme which can hydrolyze starch. The absence of starch in the area surrounding the bacteria gave the clear zone after the addition of iodine solution (Evans *et al.*, 2004).

7. CONFIRMATION OF PROTEINS IN THE CELL FREE SUPERNATANT OBTAINED FROM *Bacillus licheniformis* BY BIURET METHOD

Presence of protein in the cell free supernatant was confirmed by the formation of a bluish purple colour. Studies showed the colour change from blue to purple colour solution which indicated high concentration of proteins (Pratt *et al.*, 2011). In the present study, the concentration of protein in the cell free supernatant is low which gave a bluish purple colour on addition of biuret reagent.

8. PURIFICATION OF PROTEINS BY GEL FILTRATION CHROMATOGRAPHY

8 test tubes of 5 ml fractions for each pH was collected from the column. Each 5ml fraction eluted from the column at a particular pH was eluted in 5 minutes**.** A total of 40 minutes was taken for elution of 5ml sample into 8 test tubes at a particular pH. Some studies showed the use of sephadex for small fractionation ranges and agarose for larger molecules are employed as the matrix. The sample size was 1-5 % of the total bed volume and the protein concentration in the sample not exceeding 20 mg/ml. Maximum resolution in gel-filtration chromatography is obtained with long columns. 0.1 M NaCl or KCl was added to the eluent to avoid Van der Waals interactions. Low flow rates offering maximum resolution like 2 mL/cm^2/h can be used although much higher flow rates like 30 mL/cm^2/h can also be employed for rigid matrices (Ó'Fágáin *et al.*, 2011).

9. IDENTIFICATION OF PROTEIN CONCENTRATION BY BIURET METHOD

Fractions 5 and 6 of pH 6, fractions 5 and 6 of pH 6.5, fractions 4,5 and 6 of pH 7, fractions 4,5 and 6 of pH 7.5 and fractions 4 and 5 of pH 8 showed a colour change from blue to a slightly purple colour after the addition of biuret reagent.

10. TESTING OF ANTI-BACTERIAL ACTIVITY AGAINST *Listeria monocytogenes* and *Escherichia coli.*

The fifth fraction of pH 6.5 showed a zone of inhibition of radius 8mm against *Escherichia coli* and a zone of inhibition of 6mm against *Listeria monocytogenes*. This comparative study showed that the purified protein shows greater antimicrobial activity against *Escherichia coli* than against *Listeria monocytogenes*. It can also be inferred that a higher zone of inhibition was obtained when agar well diffusion was carried out using the purified protein fraction than the crude cell free supernatant which showed only a 4 mm zone of inhibition against *Listeria monocytogenes*.

Fig. Antibacterial activity against *E.coli*

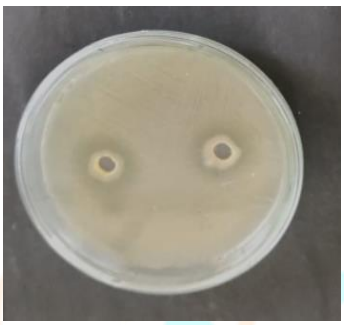


Fig. Antibactaerial activity against *Listeria monocytogenes*

11. ESTIMATION OF PROTEIN BY LOWRY METHOD

The concentration of protein in the purified sample was estimated by noting the absorbance at 660 nm. A standard curve was plotted to determine the protein concentration of the test sample which is the fifth fraction of pH 6.5 that exhibited antimicrobial activity.

The concentration of the test sample was found to be **507.9 µg/ml** by plotting the standard curve.

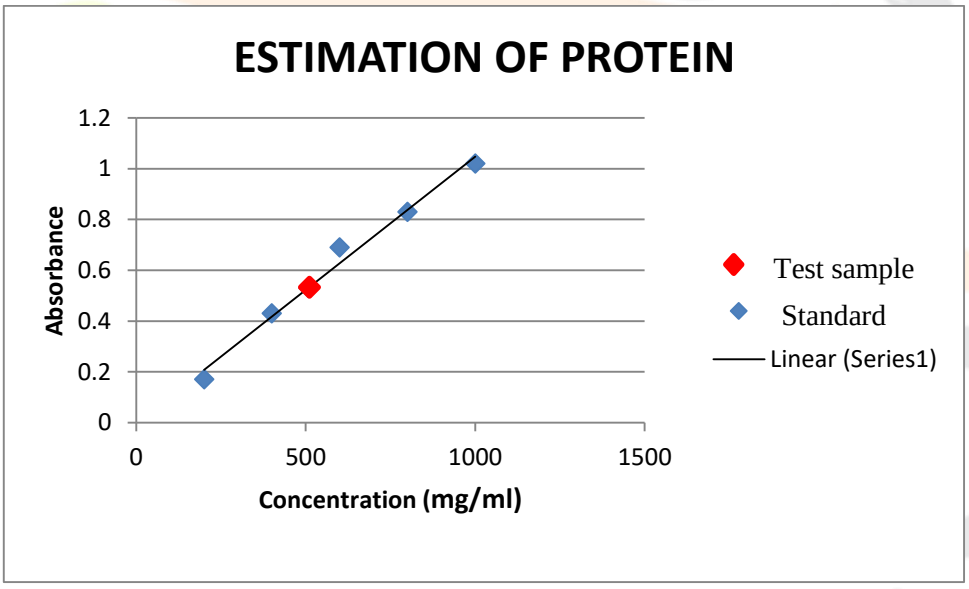
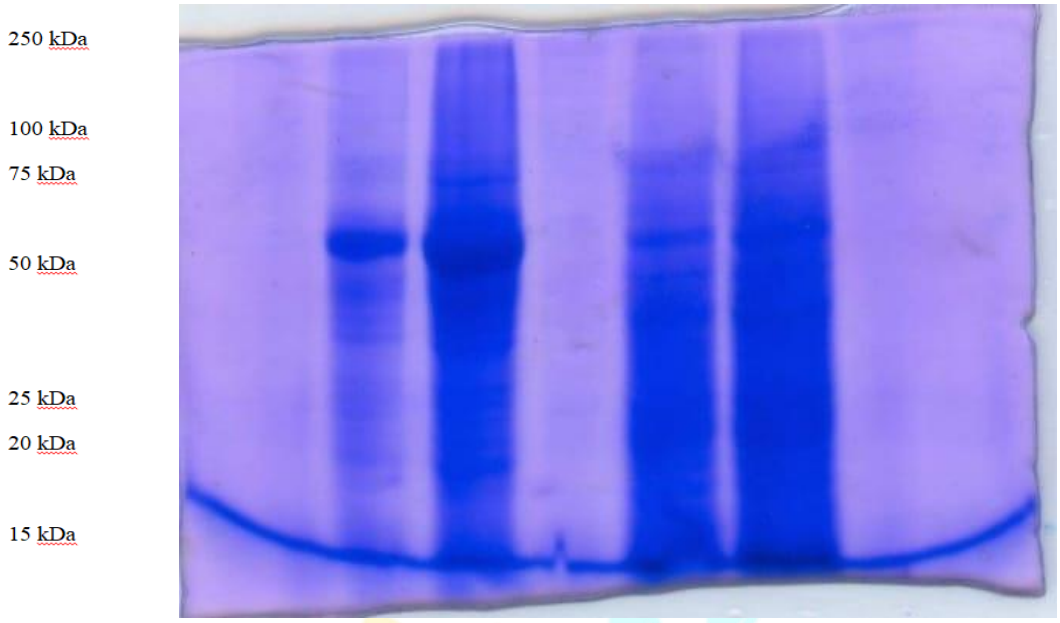


Fig. Graph for Lowry's method of protein estimation

12. SEPARATION AND CONFIRMARION OF PROTEIN USING SDS PAGE

The size chart for SDS PAGE using 10 percentage acrylamide was analyzed and found that the bands were formed in the molecular weight range of 50 to 75 kDa. The molecular weight of bacteriocins produced by *Bacillus licheniformis* range from 1.4 to 55 kDa (protein gel migration charts, Bio-Rad). So, it can be inferred that the corresponding bands produced by purified protein from the 5th fraction of pH 6.5(first band) and that of crude cell free supernatant (second band) falls in the molecular weight range of bacteriocins (Aslam *et al*., 2011). The proteins can be further subjected to amino acid sequencing or other purification techniques for the separation and purification of bacteriocins.



First well = Purified protein (5th fraction of pH 6.5)
Second well = Crude cell free supernatant
Third well = 4th fraction of pH 6.5
Fourth well = 6th fraction of pH 6.5

Fig. SDS PAGE of the purified protein

CONCLUSION

Natural antimicrobials are an alternative to the chemical and artificially synthesized substances which poses a threat to the health and well-being of living things. Bacterial sources are a great reservoir for obtaining such natural antimicrobials which can be used in food preservation as well as antibiotics. *Bacillus licheniformis* was found to be an ideal microorganism isolated from natural sources like curd that produces a wide range of antimicrobials. The inhibition of growth of *Escherichia coli* and *Listeria monocytogenes* showed that the purified proteins from *Bacillus licheniformis* can serve as natural food preservatives after safety testing. It may also be employed as biopreservatives in cosmetics and pharmaceuticals and used to control plant pathogens in agriculture.

ACKNOWLEDGEMENT

The authors are grateful to the management of Dr. N.G.P Arts and Science College, Coimbatore, the department of Biotechnology. They also wish to express their sincere thanks to DBT STAR College Scheme for the financial support rendered.

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