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PRODUCTION, EXTRACTION AND PURIFICATION OF BACTERIOCIN FROM LACTIC ACID BACTERIA AND EVALUATING ITS ANTIBACTERIAL POTENTIAL AGAINST FOOD SPOILAGE BACTERIA

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ABSTRACT:

Preservation of foods during storage is considered important step in seafood industries. Hence the aim of this research is to analyze the effect of bacteriocin as bio-preservative agents against the seafood pathogens. Biopreservation using bacteriocin from two lactic acid bacteria, *Lactobacillus acidophilus* – L1 and *Lactobacillus sporogenes* – L2 against different food pathogens were studied in the present research. Bacteriocin from Lactic acid bacteria were purified using dialysis method; and SDS-PAGE analysis was carried out to identify the protein bands of bacteriocin. Antibacterial activity of bacteriocin concentrations was evaluated using standard agar well diffusion method. SDS-PAGE results of the purified fractions of L1 and L2 showed three distinct protein bands with molecular weight of 55kDa, 30kDa, 10kDa; and five distinct bands with molecular weight of 90kDa, 76kDa, 54kDa, 24kDa and 20kDa respectively. Antibacterial activity test results of L1 bacteriocin revealed inhibitory zones ranging from 13.9mm to 19.3mm against all test bacterial cultures. And L2 bacteriocin exhibited inhibitory zones ranging from 14.9mm to 20.3mm against all test bacteria. The inhibitory effect of bacteriocins on the viable cells of food pathogens showed maximum cell inhibition. The bacteriocin exposed to *Salmonella* cells reduced maximum of about 90% followed by *Staphylococcus aureus* upto 86%. As future studies, the antibacterial action of the extracted nutraceutical compounds shall be increased by handling further purification techniques like chromatography and other methods. The hurdle factors need to be studied in detail as future study to identify the best combination factors for food preservation against food spoilage microorganisms.

KEYWORDS:

LACTIC ACID BACTERIA, NUTRACEUTICALS, BIOPRESERVATION, BACTERIOCINS, BIOACTIVE METABOLITE.

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INTRODUCTION

To avoid contamination and to maintain hygienic conditions various food products are stuffed in different packaging materials. To enhance shelf life of packed foods and to prevent them from contagious attack various methods are followed (Alamri et al., 2021). Seafoods are a type of food products that are packed in containers using various packaging methods. Seafood are highly reactive and get spoiled easily by different spoilage organisms like *Escherchia coli*, *bacillus* spp, *Shigella* spp, *Vibrio* spp, *Salmonella* spp and *Staphylococcus* aureus (Kyule et al., 2022). It is announced that microbial attack is usual in the post-processing phase in food packaging (Amit et al., 2017).

The toxins also causes damages to packed food products apart from microbes. *Staphylococcus* toxins are a kind of toxic metabolites that are built even during storage temperature causes proteolysis in packed food products (Abril et al., 2020). Other than contagious attack the food also gets affected due to various process during storage at 18^0C or less than that. The ice crystals produced in freezing conditions also cause damage to sea foods.Other results like food elimination, liquid or humidity loss, nourishment value reduction, texture and odour or physical changes are also reported (Hoa et al., 2009). Methods like (Drying, Chilling, Brine, Canning, Smoking, Freezing and Fermentation) increases the shelf-life of the packed food products. These are the various type of methods that help to prevent microbial attack and their toxic metabolite production during storage and transportation (Saini et al., 2021).

Biopreservation is the method of choice for many researchers and scientists for preserving different types of packed foods (Teneva et al., 2023). The antimicrobial compounds derived from various probiotic lactic acid bacteria is found to be highly favourable in preventing the metabolic reactions during transportation and storage (Akbar Ali and Anal, 2014). Various types of Lactic acid bacteria (LAB) engaged as biopreservatives are

Lactobacillus, Lactococcus, Pediococcus and *Enterococcus* (Khan et al., 2010). Cleveland *et al* (2001) announced that bacteriocins built by probiotic lactobacillus are hostile to other contamination causing pathogens in packed foods. The researchers also announced about several types of bacteriocins and its means of action on spoilage microbes. Bacteriocins like lactacin B, lactocin, plantaricin and helveticin have their own ability to prohibit cell wall or cell membrane combination in contagious bacteria. Bacteriocins helps in the inhibition of RNase or DNase activity in different pathogens and improves the cell membrane permeability (Galvez et al., 2007).

Hence the present work was aimed to investigate the effect of bacteriocin as antibacterial and inhibitory proteins against food spoilage bacterial species. Antagonistic activity of bacteriocin extracted from different probiotic bacterial cultures were reported to employ along with other significant hurdle factors in several research works as per literature survey (Cleveland et al., 2021). Hurdle factors with bacteriocin signifies the synergistic effect of employing different preservation methods for inhibiting the growth of food spoilage organisms (Leroi et al., 2015). Before studying the hurdle factors, the main aim of the present study was initial production, extraction and purification of bacteriocin from two different Lactic acid bacteria (*Lactobacillus acidophilus* – L_1 and *Lactobacillus sporogenes* – L_2).

Also with aim of determining the protein profile and antibacterial activity of the purified bacteriocin fractions, SDS-PAGE and standard agar well diffusion method was used respectively. Following objectives were framed to meet the above mentioned aim of the present study. To extract and purify the bacteriocin from two lactic acid bacteria, to determine the protein profile of the bacteriocin fractions, to evaluate the antibacterial activity of the purified bacteriocin fractions against different food spoilage bacteria.

MATERIALS AND METHODS

The present research work was carried out in Department of Microbiology, RVS Arts and Science College, Sulur, Coimbatore, Tamil Nadu, India. The research work was performed during the period of July 2023 to November 2023.

SELECTION OF FOOD SPOILING BACTERIA

Four different food spoilage causing organisms *Staphylococcus aureus*, *Escherichia coli*, *Bacillus* sp and *Salmonella* sp are collected from food processing laboratory in Coimbatore, Tamil Nadu, India and sub-cultured, stored under standard laboratory condition for the present research.

SELECTION OF LACTIC ACID BACTERIA AND CULTURING METHOD (SAHAR ET AL., 2017)

Lactobacillus acidophilus and *Lactobacillus sporogenes* were procured from food processing laboratory. The cultures were cultured onto MRS media. About 10ml of MRS broth was prepared and the selected strains (*Lactobacillus acidophilus* – L_1 and *Lactobacillus sporogenes* – L_2) were inoculated separately and kept in the incubator shaker for 72 hours with 180rpm at 37°C. After identifying the turbid growth of the organism, the entire 10ml culture was inoculated onto 100ml MRS broth and incubated at similar condition. After incubation period, MRS agar media was prepared and the cultures were transferred separately. Each sub-cultured plates were incubated at 37°C for 24 to 48hours and maintained in refrigeration condition after incubation period.

PRODUCTION AND EXTRACTION OF BACTERIOCIN FROM *LACTOBACILLUS* SPP (MUHAMMAD ZAHID ET AL., 2015)

Production and extraction of bioactive metabolite, bacteriocin as extracellular substance was done from the selected *Lactobacillus* spp (*Lactobacillus acidophilus* and *Lactobacillus sporogenes*). The procedure was explained briefly below. About 50 ml of MRS broth was prepared and the selected strains (*Lactobacillus* spp) were inoculated and kept in the incubator shaker for 72hours with 170 rpm and 37°C. The entire 50ml culture was inoculated onto 100ml MRS production media and incubated using the above condition. As a scale up process, the 100ml cultured bacterial cells were again transferred to 250ml production media and incubated for the production of nutraceutical bacteriocins. After incubation the broth was centrifuged and the supernatant containing the bacteriocin was collected in a clean separate flask. The concentrated bacteriocin extracts were re-dissolved in 5ml of sterile distilled water; filter sterilized and stored in sterile eppendorf tubes under refrigeration conditions.

PURIFICATION OF EXTRACTED BACTERIOCIN (GORAYA ET AL., 2013)

About 200ml of cell free supernatant was precipitated using 80% saturation level of ammonium sulphate; then it was suspended in 20mM Phosphate buffer (pH 7). Following this step, dialysis was carried out after activating the dialysis membrane. The crude protein was filled in the dialysis bag, packed and dialyzed for 12h against 20mM Phosphate buffer (pH 7) at 4°C. After dialysis, the partially purified protein fractions of bacteriocin was collected, centrifuged and the pellet was dissolved in Phosphate buffer.

SDS-PAGE ANALYSIS OF PURIFIED BACTERIOCIN (HASSAN ET AL., 2020)

Molecular weight of the extracted bacteriocin fractions of *Lactobacillus acidophilus* (L_1) and *Lactobacillus sporogenes* (L_2) was identified using the method of Hassan *et al.,* (2020). The fraction was mixed with the sample buffer containing SDS (Merck) at a ratio of 1:1 (v/v). The sample (20µg protein) was loaded onto the polyacrylamide gel made of 8% separating gel and 4% stacking gel and subjected to electrophoresis at a constant current of 50mA/gel and 120V using a Mini Protein II unit (Genei Electrophoresis, Bangalore, India). After separation, the gel was stained with 0.1% Coomassie Brilliant Blue R-250 and

destained in 25% methanol and 12% acetic acid. To estimate the molecular weight of the protein fraction, the markers including myosin from rabbit muscle, β-galactosidase from *Escherichia coli*, phosphorylase-B from rabbit muscle, bovine serum albumin, glutamic dehydrogenase from bovine liver, glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle and carbonic anhydrase from bovine erythrocytes was used in parallel.

ANTIBACTERIAL ACTIVITY OF BACTERIOCIN FROM *LACTOBACILLUS ACIDOPHILUS* (L_1) AND *LACTOBACILLUS SPOROGENES* (L_2) (MUHAMMAD ZAHID ET AL., 2015)

The antibacterial activity of extracted bacteriocin from *Lactobacillus acidophilus* (L_1) and *Lactobacillus sporogenes* (L_2) was evaluated against the test organisms by well diffusion method. All the test cultures were inoculated in a sterile Nutrient broth and allowed to attain the growth for 24 to 48 hours. Sterile Mueller-Hinton Agar plates were prepared and allowed to solidify. About 0.1% inoculum suspensions of the test organism were swabbed uniformly over the agar surface separately. Under sterile conditions, 6mm wells were cut on the agar surface of each plates. About 20µl of bacteriocin fractions (*Lactobacillus acidophilus* (L_1) and *Lactobacillus sporogenes* (L_2)) were loaded into their respective wells (three different concentrates were used and named as 1X, 2X and 3X). In parallel, 4µg/ml of streptomycin was used as standard to compare the results of the concentrates. All the plates were incubated at 37ºC for 24h. The antimicrobial activity was evaluated in terms of zone of inhibition around the wells in all the inoculated NA plates. The inhibition clear zones were measured and recorded in millimeter and compared with standard.

EVALUATING THE INHIBITORY EFFECT OF BACTERIOCINS ON THE VIABLE CELLS OF SEA FOOD PATHOGENS (WEIJIA LI ET AL., 2015)

The inhibitory effects of the bacteriocin extracts from the *Lactobacillus* spp are determined on the viable bacterial cells (*Staphylococcus aureus*, *Escherichia coli*, *Bacillus* sp and *Salmonella* sp). In brief, about 1ml of actively growing culture was taken and its absorbance indicating the growth of the organisms was measured at 600nm using a UV-Vis Spectrophotometer. To this growing culture, 1 ml of bacteriocin was added and incubated for 10 min. The above mixture was centrifuged at 8000 rpm for 5 min. Then, the pellet was collected and re-suspended in 1 ml of sterile water. The CFU was enumerated for both bacteriocin exposed and Control cells. The difference in CFU for test sample and control was calculated to express in percentage of cell inhibition (viable cells).

Cell inhibition (%) = A – B/A X 100

Where A - Control cells, and B – Bacteriocin exposed cells

RESULTS AND DISCUSSION

PURIFICATION OF THE NUTRACEUTICAL BACTERIOCINS

In the present study, *Lactobacillus acidophilus* – L_1 and *Lactobacillus sporogenes* – L_2 were cultured and maintained under sterile condition in MRS agar media (Fig. 1). The cell free culture filtrate from *Lactobacillus* sp production media was extracted and purified after scale up process (Fig. 2). The crude contents of bacteriocin purified by dialysis method based on the concept of Cintas *et al.*, (2001). The steps used for extraction, purification of bacteriocin was in accordance to the method described by Jack *et al.*, (1995)

SDS-PAGE ANALYSIS OF PURIFIED BACTERIOCIN

Molecular weight of the extracted bacteriocin fractions of *Lactobacillus acidophilus* (L_1) and *Lactobacillus sporogenes* (L_2) was analyzed. Three distinct protein bands with molecular weight of 55kDa, 30kDa and 10kDa was found evident for the bacteriocin extracted from *Lactobacillus acidophilus* (L_1). And five distinct bands with molecular weight of 90kDa, 76kDa, 54kDa, 24kDa and 20kDa was observed for the bacteriocin extracted from *Lactobacillus sporogenes* (L_2). In Fig. 3, the protein bands with its respective molecular weight compared with Marker sample was presented separately for both species. From figure, L_1 and L_2 are the respective protein samples extracted from *Lactobacillus acidophilus* and *Lactobacillus sporogenes*; and L_3 was the marker sample used to compare and find the molecular weight of the extracted bacteriocin.

INHIBITORY EFFECT OF BACTERIOCINS ON THE VIABLE CELLS

The results of the inhibitory effect of bacteriocins on the viable cells was presented in Tabel-3. During the analysis, all the pathogens exposed to bacteriocin of *Lactobacillus acidophilus* showed maximum cell inhibition. The bacteriocin exposed to *Salmonella* cells reduced maximum of about 90% followed by *Staphylococcus aureus* upto 86% after enumerating the CFU in a Plate Count Agar media. Similar inhibitory effect was found for bacteriocin extracted from another species *Lactobacillus sporogenes* (Table-4). The obtained results were found to be well in accordance to the results of antibacterial activity; where the bacteriocin in the well diffusion method showed good inhibitory zones against all the test organisms.

Bacteriocin of their broad and narrow spectrum efficacy, act as potential protein against antibiotic resistant bacteria. The mode of action of bacteriocin acts mainly on the bacterial cell membrane and cell envelope; further affects gene expression levels inside the bacterial cytoplasm (Alomari et al., 2022). The decrease in cell metabolism occurs because most antimicrobial peptides work by interacting with the bacterial cell surface, followed by disruption of cellular integrity (Nawrocki et al., 2014). The mode of action of bacteriocin was found to be well correlated with study conducted by Motta and Brandelli, (2008). In their study, the bacteriocin-like substance (BLS P34 – an antimicrobial peptide) showed good inhibitory and lethal effect on the bacterial wall of

Listeria monocytogenes (ATCC 7644).

The extended antibacterial potential of the extracted nutraceutical compounds was mainly due to their significant mode of actions as reported earlier. Montville *et al* described that bacteriocins would dissipate trans-membrane potential and increase the membrane permeability of organisms to ions. The permeability thus leads to collapse of proton motive force in the organism. The collapse will subsequently damage the plasma membrane or cell wall of the organism leading to release of all cytoplasmic constituents from the cell and cell death (Montville and Chen, 1998).

Klein *et al*., (2016) reported that different nuclease baceriocins are present in the inhibition of DNA, RNA and protein synthesis along with permease function and show the foremost effect on the position of energy by bacterium. Walker *et al*., (2007) reported that the nuclease bacteriocin delivered to the cytoplasm of a specific cell. which includes the DNA chromosomal cleavage randomly led to the cell death. Cotter *et al*., (2013) influenced that most of the bacteriocins which execute gram negative bacteria can conquer on their bacteria via interfering with proteins, RNA, DNA metabolism. Cotter *et al*., (2005) reported that bacteriolytic proteins like lysostaphin which belongs to bacteriocins class 3 directly effect on cell wall. Which is related to particular gram posistive bacteria, so cause the death and finally lysis the target cell.

Van Belkum *et al*., (1991) says that lantibiotics can collaborate with the cytoplasmic membrane of their targets and form pores. Tolpekina *et al*., (2004) reported that the formation of pores in the cytoplasmic membrane is accompanied by rise of free energy. Prince *et al*., (2016) influences that pore formation leads to rapid dispersion of transmembrane electrostatic potential which direct to the rapid death of bacterial cells. Preciado *et al*., (2016) reported that pore formation is the well-known mechanism. Where antibacterial proteins bind to the particular receptors on cells and develop pores in the membrane. Which is called as cell permeability and leads to the death of pathogenic microorganism.

Negash & Tsehai, (2020) reported that baceriocins obstruct the growth of target target organisms in various mechanisms. Some bacteriocins show antimicrobial activity by enzymatic activity, like colicin E2 showing DNase activity, colicin E3 showing RNase activity and megalin A-216 shows activity against the targeted organism. Qiao *et al*., (2021) shows that bacteriocins have bactericidal effects that may tag with or without cell lysis. Rahmeh *et al*., (2020) influenced that it is produced from LAB mostly obstructs gram positive bactria and exerts its antibacterial effect by targeting the cell envelopeassociated mechanisms.

ANTIBACTERIAL ACTIVITY OF BACTERIOCIN

The results of antibacterial activity of bacteriocin extracts showed good activity against all the food pathogens. In Table-1 and Fig. 4, the antibacterial inhibitory zones obtained for bacteriocin from *Lactobacillus acidophilus* was presented. In Table-2 and Fig. 5, the antibacterial inhibitory zones obtained for bacteriocin from *Lactobacillus sporogenes* was presented. Interestingly, for both type of bacteriocin fractions, 3X concentrate exhibited more inhibitory zones when compared to 1X and 2X concentrates; and slightly lesser than the standard (Streptomycin).

The obtained results when compared to the literature survey found supportive due to mode of action of bacteriocin on cell components of bacteria. Islam *et al.,* (2020) determined antibacterial activity of bacteriocin protein against six isolates. The inhibitory zones were ranged from 20mm to 35mm against the isolates. Elayaraja *et al*., (2014) extracted bacteriocin from Lactobacillus murinus and found that purified compound exhibited broad inhibitory spectrum against Gram-Positive and Gram-Negative bacterial isolates ranging from 13.5mm to 28mm zone size. Deshmukh and Thorat (2018) extracted purified bacteriocin from *Lactobacillus brevis* and *Lactobacilllus zymae* and determined antibacterial activity against *Bacillus coagulans*; the researchers found zone size ranging from 7mm to 16mm against three strains of *B. coagulans*. Nivedita and Neha (2008) studied the antibacterial activity of bacteriocin from *Bacillus mycoides* after isolating from whey. The researchers found inhibitory zones ranging from 10mm to 30mm against different *Listeria* species.

The literature reports thus found supportive to our present findings in terms of antibacterial activity of bacteriocin influencing its mode of action to damage the cell components of bacteria. The results concludes that bacteriocin extracted in the present study shall be utilized as antibacterial substance and also as biopreservative in food during storage conditions.

CONCLUSION

Antibacterial neutraceutical bacteriocins were extracted and purified from two different Lactic acid bacteria in the present study. Partially purified fractions showed distinct protein bands during SDS-PAGE analysis. Potential antibacterial action was found evident against different food pathogens based on the inhibitory zones obtained for bacteriocins extracted from two different lactic acid bacteria. Also, its higher thermostability, wide pH tolerance, bactericidal mode of action and proteolytic nature, it could be used as a potent natural preservative to enhance shelf life of processed foods. As future studies, the antibacterial action of the extracted nutraceutical compounds shall be increased by handling further purification techniques like chromatography and other methods. The hurdle factors need to be studied in detail as future study to identify the best combination factors for food preservation against food spoilage microorganisms.

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CONFLICT OF INTEREST

Authors declare no conflict of interest in the present study.

AUTHOR'S CONTRIBUTION

First author contributed in performing all laboratory works. Second author provided all protocols to perform the laboratory experiments.

FUNDING

No funds granted.

ETHICS STATEMENT

No animals and humans used in the present research

TABLE-1: ANTIBACTERIAL ACTIVITY OF BACTERIOCINS EXTRACTED FROM *LACTOBACILLUS ACIDOPHILUS* (L1)

S. No	Test organisms	Zone of inhibition (in mm)			
		1x	2x	3x	S*
1	*Staphylococcus aureus*	0	0	16.3 ± 1.05	18.6 ± 0.75
2	*Salmonella* sp	0	09	14.6 ± 0.75	19.3 ± 1.05
3	*Bacillus* sp	0	0	15.3 ± 1.05	18.3 ± 1.05
4	*Escherichia coli*	0	10	10.9 ± 0.57	13.9 ± 0.57

*S: Streptomycin (antibiotic)

TABLE-2: ANTIBACTERIAL ACTIVITY OF BACTERIOCINS EXTRACTED FROM *LACTOBACILLUS SPOROGENES* (L_2)

S. No	Test organisms	Zone of inhibition (in mm)			
		1x	2x	3x	S*
1	*Staphylococcus aureus*	0	0	19.6 ± 0.75	20.3 ± 1.05
2	*Salmonella* sp	0	0	14.9 ± 0.57	15.6 ± 0.75
3	*Bacillus* sp	0	0	16.3 ± 1.05	20.3 ± 1.05
4	*Escherichia coli*	0	0	10.9 ± 0.57	14.9 ± 0.57

*S: Streptomycin (antibiotic)

TABLE-3: INHIBITORY EFFECT OF BACTERIOCINS OF *LACTOBACILLUS ACIFOPHILLUS* ON THE VIABLE CELLS

S. No	Test organisms	Percentage of cell inhibition (%)
1	*Staphylococcus aureus*	86.9 ± 0.57
2	*Salmonella* sp	90.3 ± 1.05
3	*Bacillus* sp	85.9 ± 0.57
4	*Escherichia coli*	82.6 ± 0.75

TABLE-4: INHIBITORY EFFECT OF BACTERIOCINS OF *LACTOBACILLUS SPOROGENES* ON THE VIABLE CELLS

S. No	Test organisms	Percentage of cell inhibition (%)
1	*Staphylococcus aureus*	85.3 ± 1.05
2	*Salmonella* sp	91.6 ± 0.75
3	*Bacillus* sp	83.3 ± 1.05
4	*Escherichia coli*	80.9 ± 0.57

Lactobacillus acidophilus **(L1)**

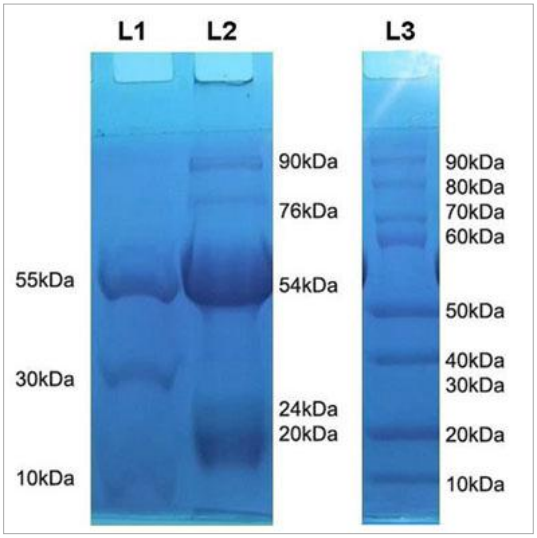
Lactobacillus sporogenes **(L2)**

FIG. 1: CULTURING THE BACTERIOCIN PRODUCING *LACTOBACILLUS* SPP

Lactobacillus acidophilus **(L_1)** *Lactobacillus sporogenes* **(L_2)**

Extracted bacteriocin from two lactic acid bacteria

FIG. 2: NUTRACEUTICAL BACTERIOCIN PRODUCTION - LABORATORY SCALE UP PROCESS

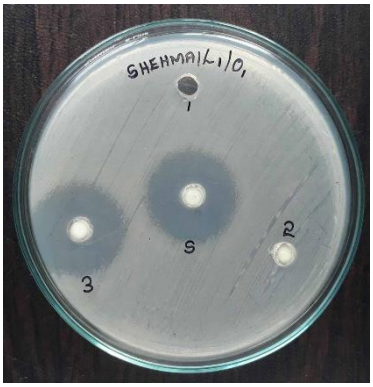


L_1 - *Lactobacillus acidophilus,*

L_2 - *Lactobacillus sporogenes*

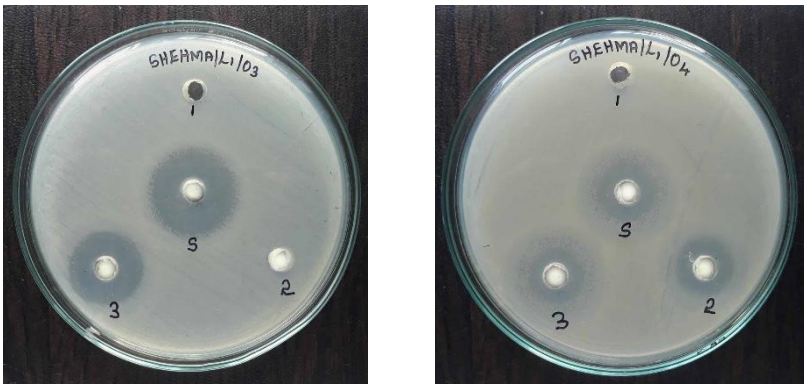
L_3 - Marker

FIG. 3: SDS-PAGE ANALYSIS OF BACTERIOCIN



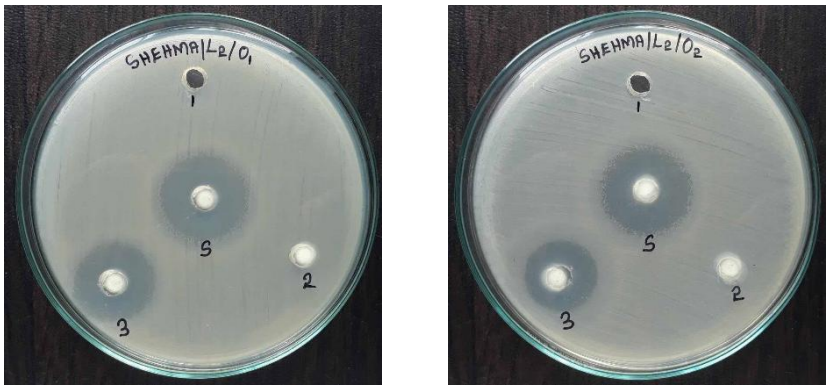
Staphylococcus aureus

Salmonella sp

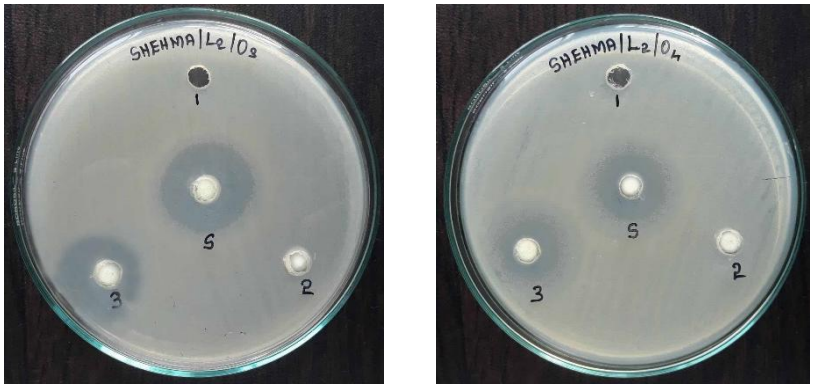


Bacillus sp *Escherichia coli*

FIG. 4: ANTIBACTERIAL ACTIVITY OF BACTERIOCINS EXTRACTED FROM *Lactobacillus acidophilus* (L_1)



Staphylococcus aureus *Salmonella* sp



Bacillus sp *Escherichia coli*

FIG. 5: ANTIBACTERIAL ACTIVITY OF BACTERIOCINS EXTRACTED FROM *Lactobacillus sporogenes* (L_2)

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