CHARACTERIZATION OF ANTIBACTERIAL ACTIVITY OF BACTERIOCIN PRODUCING LACTIC ACID BACTERIA ISOLATED FROM TRADITIONAL INDIAN FERMENTED FOOD

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Abstract

It is well known that the coming generation depend on that food product which help them to fight against infection and make them immune or healthy. Because a healthy mind stay in healthy body that's why the study towards traditional Indian fermented food products has been carried out. The present study regarding characterization of LAB and antibacterial activity of bacteriocin will be very helpful towards traditional Indian fermented food especially curd for human welfare and darolac powder for infant. Bacteriocin considered as safe (GRAS) and used in antibiotic to inhibit the pathogenic growth of micro-organisms. In the end, study elucidating their probiotic and beneficial properties may cover the way for commercial preparation of curd and darolac, biological preservation of food product and also biomedical application.

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DOI: - 10.48047/ecb/2023.12.si10.00360

Introduction

India has a rich tradition of fermented foods, which are an integral part of the cuisine in many regions of the country. Particular the most popular traditional Indian fermented foods include Idli and Dosa, Dhokla, Yogurt, Kanji, Kombucha, Pickles and Appam. Lactic acid bacteria (LAB) are commonly found in fermented foods and are known for their ability to produce bacteriocins, which are antimicrobial peptides that inhibit the growth of other bacteria (Kormin *et al*; 2001, Girma and Aemiro; 2021). LAB produce fatty acids, bacteriocins, diacetyl, organic acids, hydrogen peroxide, and other chemicals. In contrast to antibiotics, bacteriocins have a distinct digestive system breakdown mechanism and the potential to suppress the development of sensitive harmful bacteria (Salehi, 2012; Hawaz, 2014). Traditional Indian fermented foods are a rich source of LAB and have been shown to possess antibacterial activity due to the presence of bacteriocins (Pal *et al*; 2005; K Jeevaratnam *et al*; 2005). Bacteriocins have the potential to be used as natural food preservatives to improve the safety and shelf life of food products. Study isolated LAB from the fermented milk product, dahi, and found that the LAB produced a bacteriocin that was active against several pathogenic bacteria and several food spoilage bacteria, including *Pseudomonas aeruginosa*, *Bacillus subtilis* (Karami *et al;* 2017), *Staphylococcus aureus, Escherichia coli*, *Salmonella typhimurium* (Maqsood et al., 2008), *Bacillus cereus* and *Listeria monocytogenes* (Jones et al., 2008; Khandare and Patil; 2015)

Material and Methods

Isolation of *Lactobacillus* spp.

Curd is taken in sterilized flask. Under the aseptic conditions curd was serially diluted from 10-2 to 10 -5 from this dilutions 10-4, are selected. Spread plate technique further with streak plate technique is done on MRS medium. They are incubated in incubator 370C which is optimum temperature for *Lactobacillus* broth. Incubation at 370C for 24 hrs. Broth after 24-48 hrs shown *Lactobacillus* species growth and these species for 24 hours. After the period of incubation, the specific isolated colonies were grown. Colony characterization is done for this colonies found to be Lactobacillus species. One colony shows 100% resemblance with Lactobacillus acidophilus. The isolated colony formed on the MRS agar plates was identified using gram stain, biochemical tests. The identification was performed according to Bergey's manual of determinative of bacteriology. The culture was kept in MRS agar slant and stored at 4 0C for long term storage.

Biochemical characterization of the isolated bacterial strain

Identification of the isolated bacteria as *Lactobacillus* species was performed according to their morphological, cultural, and physiological and biochemical characteristics by the procedures as described in Bergey's Manual of Systematic Bacteriology. The tests carried out were Gram staining, Capsule staining motility test, production of catalase, Indole, Methyl Red, Voges Proskauer, Citrate, Starch Hydrolysis, endospore test, milk coagulation activities and NaCl and phenol tolerance test.

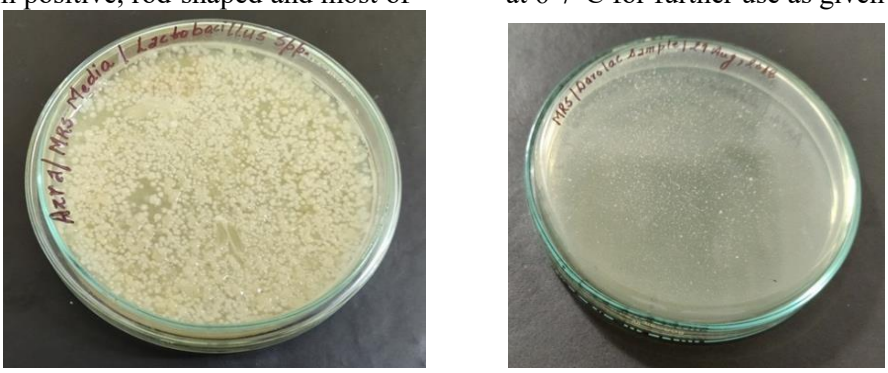
Sugar Fermentation Test, Catalase Test, Indole Production Test, Methyl Red Test, Vogues Proskauer Test, Citrate Utilization Test

Result and Discussion: - The present study on characterization of antibacterial activity of bacteriocin producing LAB from traditional fermented food was carried out to isolate lactic acid producing bacteria and its Identification. Present study also emphasizes to screen production of bacteriocin by fermentation media and purify the crude protein from the mixture of enzymes followed by characterize the antimicrobial potential and range of activity of protein against the pathogenic bacteria.

Isolation and Identification of Lactobacillus spp. from fermented food: - De Mann Rogosa Sharpe (MRS) Agar was reported to be the best suitable selective media for the isolation of *Lactobacillus* spp. (lactic acid bacteria) by **(Ghoddusi, 2002)**. Selective medium is used to cultivate specific type of microorganisms. Thus, the ability of bacterial species to be cultured on specific media is regarded as an important characteristic in identification of the microorganisms.

Sample of fermented food like curd were collected and transported to the laboratory for the isolation of Lactic acid bacteria. Streaking was done with the help of sterile swab on the surface of MRS agar. The plates were incubated at 37°C in an incubator. The plates were examined for the growth of micro-organisms after 24 hours. Mucoid and whitish colonies were observed. Picked colony and re-streaked on the surface of MRS agar to purify the culture. Well isolated colonies were once again

streaked to make sure that the culture is pure as shown in Figure-1.All the isolates which were found to be gram positive, rod-shaped and most of them were non-motile were selected and transferred on MRS slants and stored in refrigerator at 6-7°C for further use as given in the figure 2.



A B

Figure:1: Isolation of *Lactobacillus spp.* (A) Curd sample and (B) Darolac sample

Phenotypic identification of the isolated pure culture was carried out on the basis of its cultural, morphological and biochemical characteristics (Table 4.5) by the procedure described in Bergey's manual (Williams and Wilkins, 1984) and (Collins and Lyne, 1980).

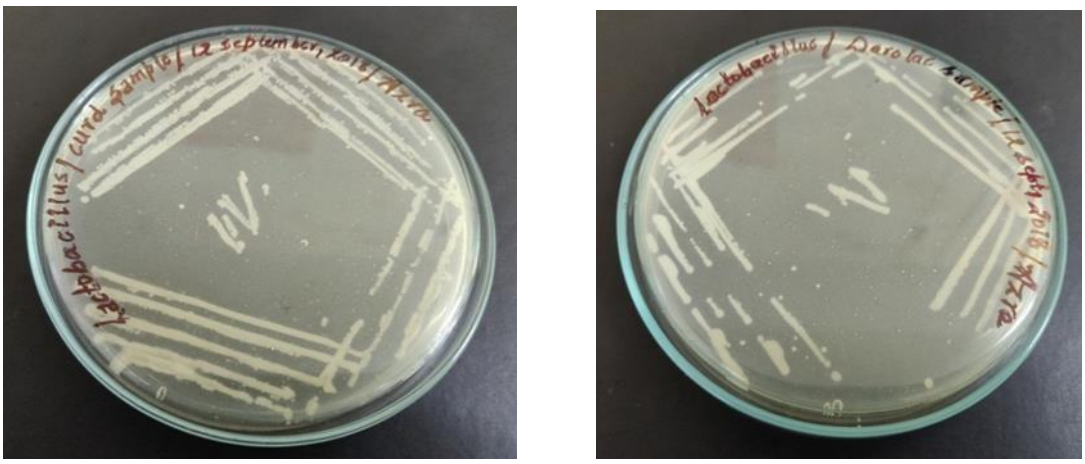


Figure 2: Growth of Lactobacillus on MRS (A) Curd sample (B) Darolac sample

By isolating on MRS agar, small circular, mucoid and whitish colonies were obtained. The colonies were further taken for gram staining that resulted in purple colored and gram positive rods. These were further checked for motility in which the isolates were negative for motility.

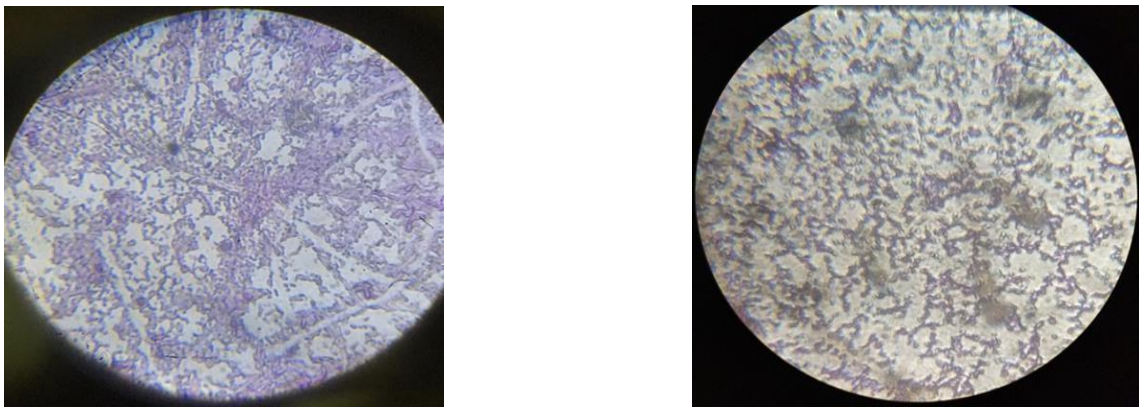


Figure 3: Gram staining of Lactobacillus on MRS (A) Curd sample -Gram positive (B) Darolac sample- Gram negative

Another investigation on other sources for isolation of lactic acid bacteria has been successfully reported. Isolation of six *Lactobacillus* spp., one bacteriocin producing strain *Lactobacillus fermentum* UN01 and 35 lactic acid bacteria was isolated from other products like Mango pulp, Chick intestine and different other sources origins respectively **(Ravi *et al.,* 2011; Udhayashree *et al.,* 2012).** Findings in the present study was found similar to other studies results reported by several other researchers where the incidence of *Lactobacillus* spp. was found as low as 30-38% as reported **(Cheriguene *et al.,* 2006; Kumari and Garg, 2007; Abd El Gawad *et al.,* 2010)** and a higher % as high as of 44-50% was reported **(Guessas and Kihal, 2004; Aziz *et al.,* 2009; Elgadi *et al.,* 2008; Ali, 2011).**

Table 1: Identification Table

Table: Morphological Characteristics			
Characteristics	***Lactobacillus* spp.**	***Lactococcus* spp.**	***Streptococcus* spp.**
Colony Morphology	White, small, circular, entire margin, small to pin point, Smooth, Glistening	Creamy, round, entire margin, small to medium colony	Whitish, creamy, translucent
Gram Reaction	Gram +ve	Gram +ve	Gram +ve
Cell Morphology	Long Rods in pairs and chains, straight	Cocci, spherical or ovoid cells in pairs or chains	Cocci, (spherical) pair or chain

Similarly another study reported about isolation and identification of higher incidence than the present study *i.e.,* 6 (26.09%) *Lactobacillus casei* (D'Aimmo *et al.,* 2007). While, others repoted the presence of *Lactobacillus casei*, reported from butter, paneer, milk, curd, yoghurt, yoghurt milk and pharmaceutical products. (Anas *et al.,* 2008; Saranya and Hemashenpagam, 2011 and Nama *et al.,* 2013). In other study *Lactobacillus plantarum* was isolated from milk, butter, milk, curd, paneer and yoghurt samples (Anas *et al.,* 2011; Sarany and Hemashenpagam, 2011; Chowdhuri *et al.,* 2012 and Nama *et al.,* 2013). Other researchers also reported the incidence of *Lactobacillus acidophilus* between the range of 12-16%, similar to the present study (Kumar and Garg, 2007; Jayalalitha *et al.,* 2009; Aziz *et al.,* 2009; Singh and Sharma, 2009; Tambekar and Bhutada, 2010). The higher percentage incidence of *Lactobacillus delbrueckii* subspp. *bulgaricus* (47.82%) was reported from yoghurt, yoghurt milk and pharmaceutical products (D'Aimmo *et al.,* 2007). Another, study reports isolation of *Lactobacillus delbrueckii* from yoghurt (fresh curd) Morami *et al.,* (2013).

Biochemical Identification:

Sugar Fermentation Test: In sugar fermentation test, isolates of *Lactobacillus* spp. gave positive result as they show gas and acid production against lactose, sucrose and glucose but some strains of *Lactobacillus* spp. gave negative result against malibiose, raffinose and xylitol.

Catalase Test: In catalase test, isolates of *Lactobacillus* spp. gave negative result as they do not show bubble formation on the addition of hydrogen peroxide.

Indole Production Test: By the addition of Kovac's reagent after 24 hours, if red color appeared then positive result and if they do not show red color then negative result. Isolates gave negative result as they do not show red color appearance on the addition of Kovac's reagent.

Methyl Red Test: Additon of methyl red in the incubated test tubes show red color ring formation which indicates positive result and the negative result observed when there is no ring color formation occur. In methyl red test, isolates of *Lactobacillus* spp. gave red coloration or the positive result on the addition of methyl red.

Vogues Proskauer Test: Addition of VP I reagent and VP II reagent represented by the development of red color indicating the presence of diacetyl, the oxidation product of acetoin, show positive result and if the black color observed then it should be negative. All the isolates show negative result for this as most of the isolates were positive for methyl red test. Therefore, *Lactobacillus* spp. are VP negative.

Citrate Utilization Test: The growth will be visible on the slant surface and the medium will be an intense Prussian blue. The alkaline carbonates and bicarbonates produced as byproducts of citrate catabolism that raise the pH above 7.6, causing the bromothymol blue to change from the original

green color to blue indicated positive result. If there is no change in color then negative result observed. After 24 hours of incubation on simmon's citrate agar, most of the isolates were citrate negative as they do not gave blue color after incubation.

Table 2: Biochemical Characteristics

Biochemical Test	Observation	Result
Sugar fermentation test	Sucrose/ Glucose/ Lactose	Positive
Catalase test	No bubble formation	Negative
Indole production test	No red color appeared	Negative
Methyl red test	Red color appeared	Positive
Vogues proskauer test	No color appearance	Negative
Citrate utilization test	No blue color appeared	Negative

DNA Isolation:

Bacterial DNA isolated by using AGE and visualization done by Gel Doc. system where 2 bands observed from sample 1 i.e. curd and 3 bands observed from 2nd sample i.e. Darolac's powder. By using spectrophotometer DNA was quantified and the result revealed that the isolated DNA exhibited $A_{260/280}$ ratio of approximately 1.4 indicating that it was satisfactory. Methods of extracting DNA from food have focused on the detection of particular microbes, including spoilage bacteria (**Wieling *et al.*, 2011 and Hosseini *et al.*, 2012**) pathogenic bacteria **(Dobhal *et al.*, 2014 and Martinon *et al.*, 2012**) and some bacteria dominant in fermented foods.**(Mamlouk *et al.*, 2011)** DNA yield and purity and PCR amplification products are usually used to evaluate the efficiency of DNA extraction methods for detection of a certain bacteria in the food matrix.**(Pirondini *et al.*, 2010**) However, to investigate the microbial communities in fermented foods, DNA extraction methods should be suitable for a wide range of microorganisms. Microbial profiles might be important for the evaluation of DNA extraction methods for microbial community analysis.

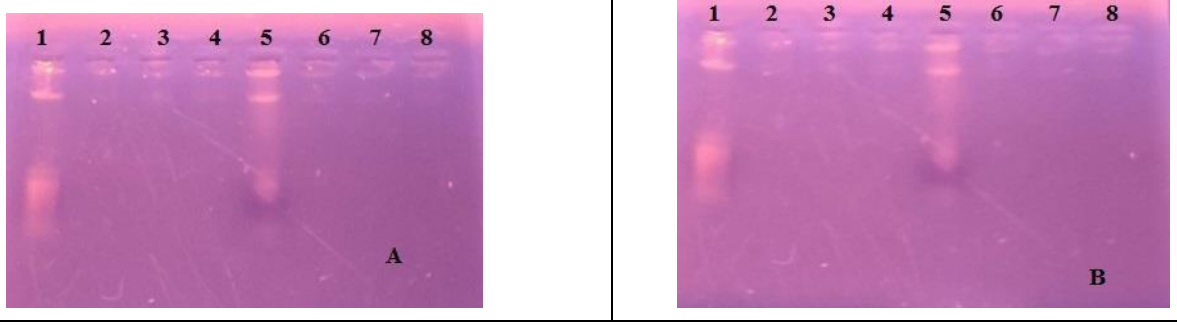


Figure 4: DNA Extraction (A) Curd sample and (B) Darolac sample

In the above images result indicate that, the sample A and sample B gel contained 0.5mL 1X TAE buffer, 24.5 mL distilled water and 0.2 gm agarose. After oven heating, 1µL EtBr was added. 5µL tracking dye and 6µL sample was loaded in both the gel. Run on electrophoretic unit and the result observed at gel doc. system. In sample A, 1 and 4 lane have genomic DNA and in sample B, lane 1 and 5 have genomic DNA.

PCR:

Polymerase chain reaction (PCR) was used for molecular identification. PCR targeting the 16S rRNA gene confirmed the high proportion of lactobacilli in the community of *Lactobacilli.*

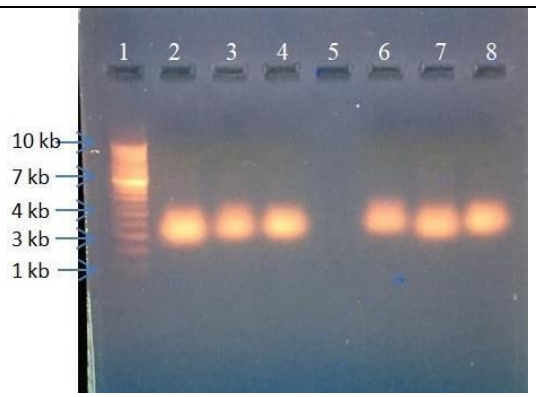


Figure 5: DNA Sequencing

In the above images, result indicate that the sample A and sample B gel contained 0.5mL 1X TAE buffer, 24.5 mL distilled water and 0.3gm agarose. After oven heating, 1µL EtBr was added. 5µL tracking dye and 6µL sample was loaded in both the gel. Run on electrophoretic unit and the result observed at gel doc. system. The 1st image confirmed the presence of genomic DNA and 2nd image indicated the amplified product with universal primer of 16S rRNA, the size of amplicon indicated in the gel image as corresponding to ladder is approx. 1.8-2.8 kb. Lane 2, 3, 4 shows PCR product of sample 1 while lane 6, 7, 8 shows PCR product of sample 2 and lane 1- 1kb ladder.

Bacteriocin production and Antibacterial activity:

By preparing fermentation media, after 3 to 4 days supernatant collected in falcon tube. Refrigerate bacteriocin and then check the antibacterial activity. The *Lactobacillus* spp. isolated having broad spectrum of antibacterial effect (maximum zone of inhibition) was extracted and partially purified and thereafter, it was again analyzed for the evaluation of antibacterial effect. The antibacterial effect of crude bacteriocins of isolates was determined by agar well diffusion method. The antibacterial effect of culture supernatant of the isolated strains of *Lactobacillus* spp. was screened and evaluated by testing against 2 selected pathogenic bacteria namely, *Klebsiella* spp. and *Escherichia coli*. Further, the bacteriocin from the strains was isolated, purified and characterized. The antibacterial activity test revealed that the isolates showed inhibition zone against *Klebsiella* spp. but no inhibition zone was observed in case of *E. coli*. The application of such a broad spectrum bacteriocin or the producer strain could result in improvement of the starter cultures of dairy products and also in food preservation. Other researchers have also reported the antagonistic activity of Lactic acid bacteria strains isolated from various foods and dairy product against *Bacillus cereus* **(Puttalingamma *et al.*, 2006).** *Lactobacillus fermentum* showed inhibitory activity against *Escherichia coli, Salmonella typhi, Bacillus cereus, Staphylococcus aureus, Pseudomonas mirabilis, Klebsiella pneumonia* and *Pseudomonas aeuroginosa*. **(Udhayashree *et al.*, 2012)** Capacity of effective bacteriocins production by *Lactobacillus* spp. is an indication that the bacteria can be used as probiotic and as bio preservative**.**

Figure 6: Antibacterial activity of bacteriocin

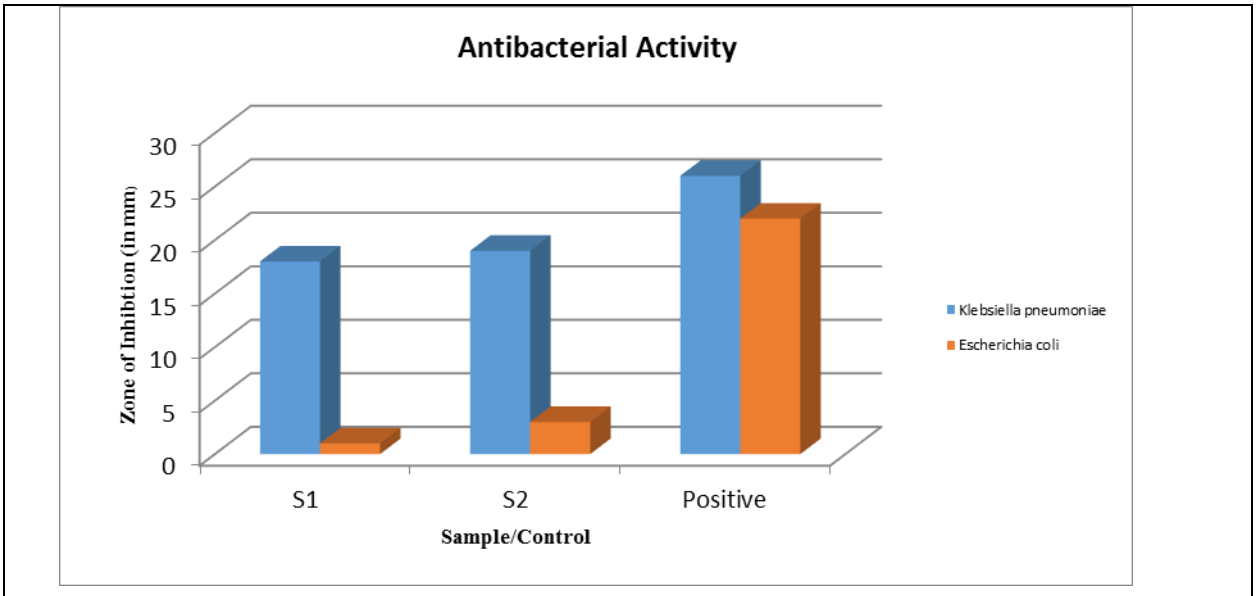


Figure 7: Antibacterial Activity

Purification of Antibacterial Compound:
Ammonium sulfate Precipitation:
Bacteriocins of lactic acid bacteria have been widely studied in recent years; however, there are relatively few studies that describe their chemical structure. This may be due to the many challenges associated with the purification of these antagonistic peptides **(Mackay *et al.*, 1997).** Various strategies for the purification of bacteriocins from cultivation broths have exploited their cationic and hydrophobic characteristics **(Cheigh *et al.*, 2004).** The purified bacteriocin exhibited complete inactivation of antimicrobial activity when treated with trypsin and pepsin. The purified bacteriocin exhibited broad inhibitory spectrum against both gram positive and negative bacteria.

Quantitative estimation of Protein using Lowry Method: The result revealed that the LAB isolates showed the presence of protein content and the quantitative estimation was carried out via spectrophotometer with OD value read at 650nm.

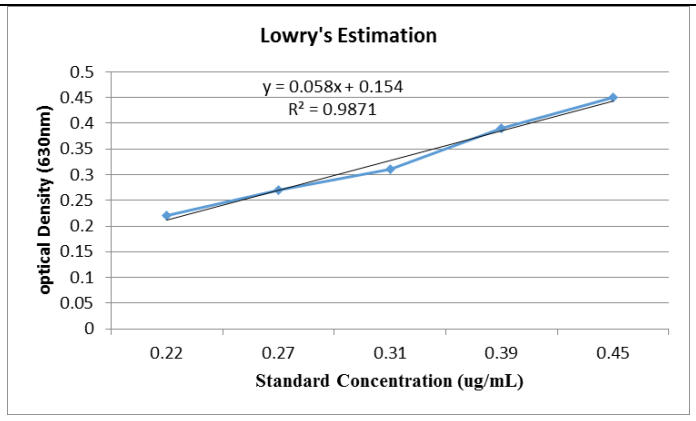


Figure 8: Estimation of Protein by Lowry Method

The equation is: Y= 0.058X + 0.154 and R^2= 0.9871
Value of X for sample 1= 6.31µg/mL and for sample 2= 7.344µg/mL

Thin Layer Chromatography After exposure to ninhydrin, pink color spot similar to the standard amino acid were observed. And the Rf value of sample 1 isolates and sample 2 isolates was determined.

$$\textit{Distance travelled by the solute from the origin}$$

Rf value=

$$\textit{Distance travelled by the solvent from the origin}$$

Rf value of sample 1:
a. Rf= 0.15
b. Rf= 0.66

Rf value of sample 2:
a. Rf= 0.71
b. Rf= 0.25

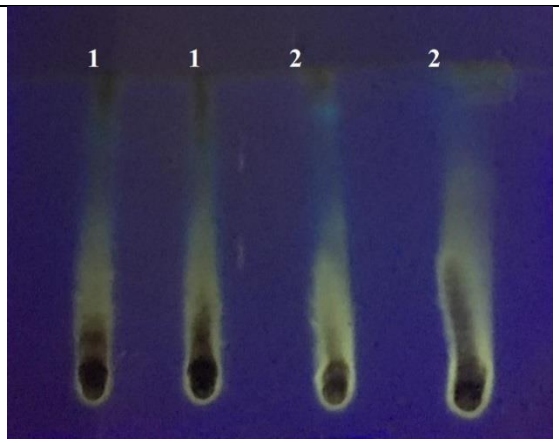


Figure 9:Thin layer Chromatography

The qualitative analysis of antimicrobial compounds by TLC compared with others experiment which also showed pink color spot similar to the standard amino acids observed **(Saranya, S., & Hemashenpagam, N. 2013).** It is also proved that thin layer chromatograms, method previously used for detecting antibacterial and antifungal substances for control of pathogens was useful to reveal the active fraction **(Vodnar *et al.*, *2010*).**

Column Chromatography: Following the removal of gross contaminants from the crude extract, remaining protein must be resolved. The most popular and ubiquitous technique for this separation is column chromatography. Column of ion exchange resins have received particular attention because they were shown to yield quantitative results with amino acids and hence would be expected to separate peptides quantitatively as well **(Moore *et al.*, 1956)**. The sample was purified and the purified sample collected at 1 minute. The result indicated that the purification is completed by column chromatography.

HPLC Profile of Bacteriocin:

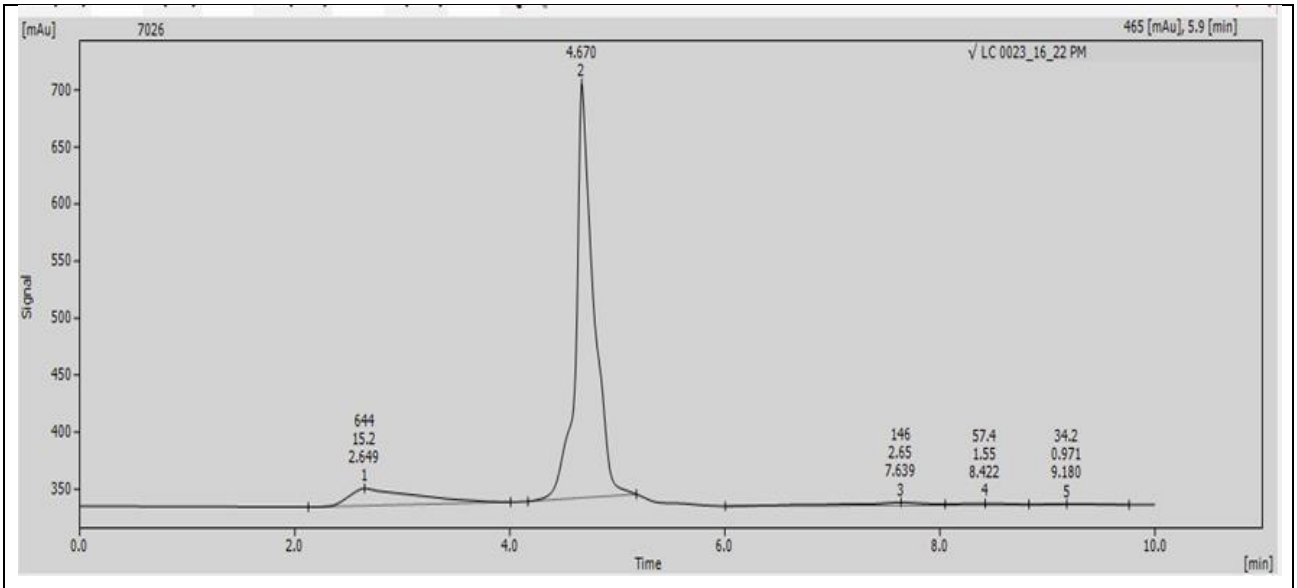


Figure 10: HPLC of Bacteriocin

	Compound Name	Peak Style	Retain [min]	Area [mAu.s]	AreaPer [%]	Height [mAu]	Amount [-]
1		Single	1.567	18.080	0.308	1.097	18.080
2		Single	2.669	711.193	12.128	18.714	711.193
3		Overlap	4.618	3886.4...	66.277	334.172	3886.4...
4		Overlap	4.857	1063.0...	18.129	126.897	1063.0...
5		Overlap	7.597	114.718	1.956	2.272	114.718
6		Overlap	8.367	43.088	0.735	1.247	43.088
7		Overlap	9.128	27.344	0.466	0.862	27.344
	Total			5863.8...	100.000	485.260	5863.8...

Represents the HPLC profile of calculated amount of Bacteriocin in the content

Bacteriocin calculated amount		
Compound Name	**Area Percentage**	**Amount**
Bacetriocin	66.277	3886.4 µg/dL

The collected amount of bacteriocin is 3886.4µg/dL. The HPLC performed for LAB and the sample was made to run for 10-15 min. volume of sample taken for analysis is 10µL and the analysis was made at 200-320nm. Methanol was used as the solvent system for the study. The number of chromatographic techniques varies from each other for bacteriocin purification in the present study in which antibacterial activity was recovered after simple precipitation with ammonium sulfate from cell free culture supernatant fluid. HPLC step of the purification procedure led to the isolation of a single active fraction having antibacterial activity **(Vodnar *et al., 2010*).**

Multiple Sequence Alignment

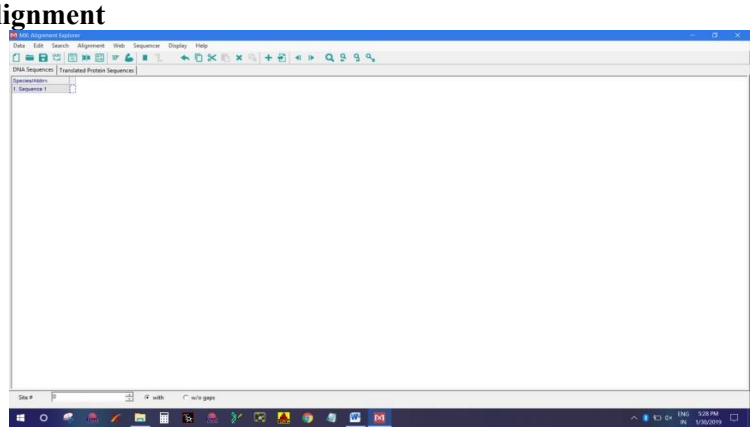


Photo 33: *Alignment home page*

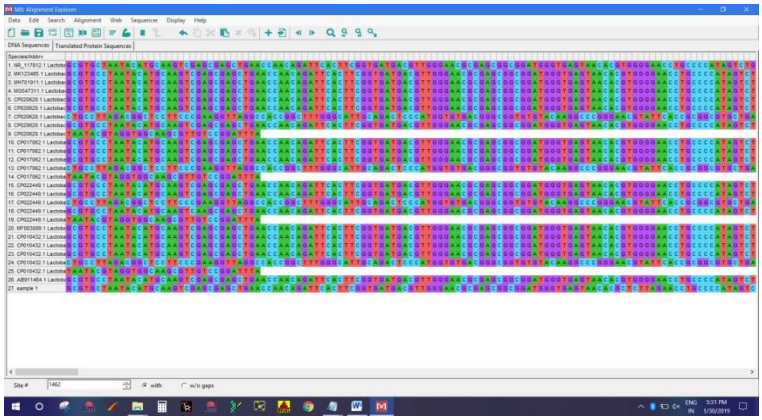


Photo 34: *Similar sequences with query sequence (sample 1)*

Photo 35: Aligned sequences (sample 1)

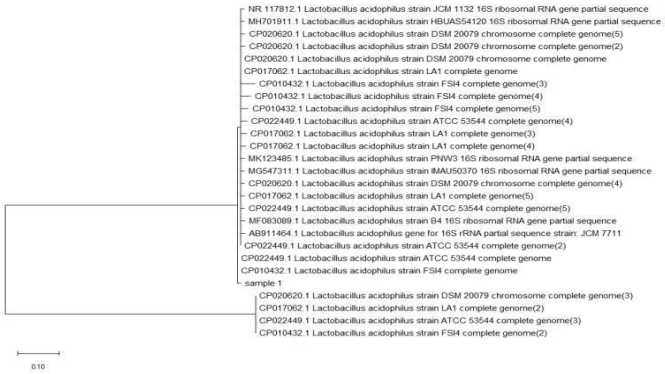


Photo 36: *Phylogenetic Tree (Sample 1*

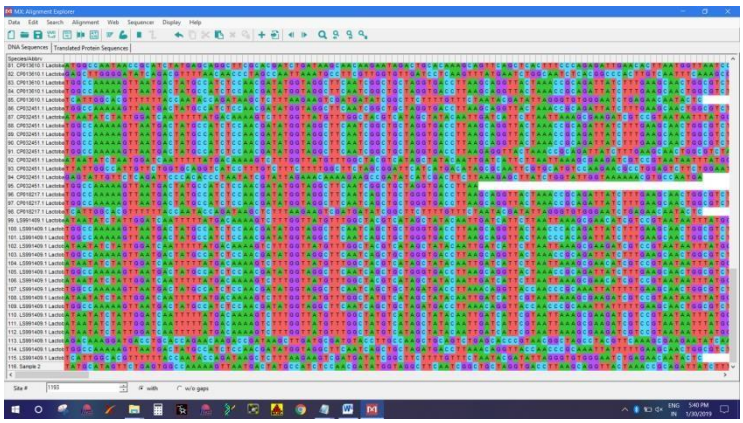


Photo 37: Similar sequences with query sequences (sample2)

Photo 38: Aligned sequences (sample 2)

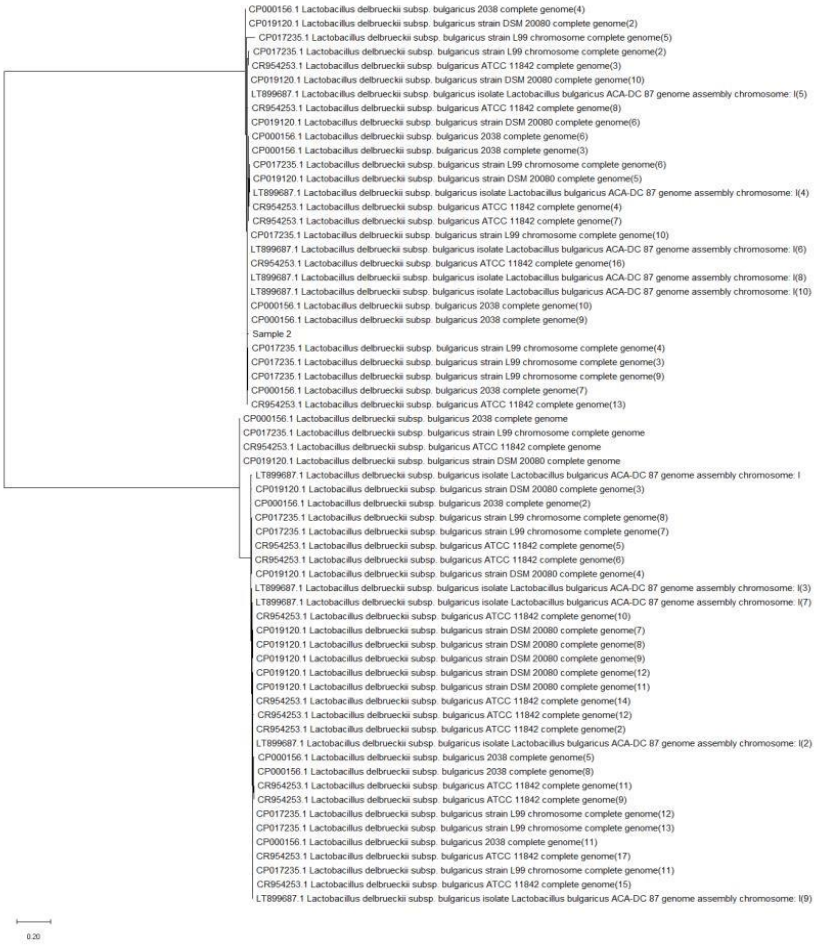


***Photo 39**: Phylogenetic Tree (Sample 2)*

16S ribosomal RNA (16S rRNA) is a component of the 30S small subunit of prokaryotic ribosomes. The genes coding for it are referred to as 16SrDNA and are used in reconstructing phylogenies, due to the slow rates of evolution of this region of the gene. The **16S rRNA** gene is used as the standard for classification and identification of microbes, because it is present in most microbes and shows proper changes.

In addition to highly conserved primer binding sites, **16SrRNA** gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for identification of bacteria **(Pereira *et al*., 2010).**

In this study **16SrRNA** detected in **45 (95.74%/)**out of **47** *S. aureus* samples. The expression no. of species **5322YPMF013** and the query id is lcl|Query_118403 that shows 97% similarity between species isolated from the sample of curd. The expression no. of species is **536JFT0301N** and the query id is lcl|Query_118439 that shows 96% similarity between the species isolated from the sample of darolac powder.

16SrRNA sequencing was done and the electrophore gram shows the sequences are of good base quality. The sequence derived from the electrophore gram was BLAST searched for similarity sequences at the NCBI (National Center for Bioinformatics) gene database.

The sequences were aligned and compared with those stored in Gene Bank (htt://www.ncbi. hlm. nih. Gov/gene bank/) by using BLAST alignment software (NCBI) and phylogenetic three was constructed.

The result obtained shows the aligned sequences with the query sequence. The sequences with maximum identity were selected to construct the phylogenetic tree. The species isolated from curd are *Lactobacillus plantrum, Lactobacillus bulgaricus* and from Darolac are *Lactobacillus acidophilus and Streptococcus* spp.

Conclusion

The present study regarding characterization of LAB and antibacterial activity of bacteriocin will be very helpful towards traditional Indian fermented food especially curd for human welfare

and darolac powder for infant. It showed that both the samples were beneficial for human welfare which improved the metabolism of human beings and provide immunity towards infectious diseases. Molecular characterization also performed to confirm the bacteriological identification. Also check the ability that the isolates utilized carbohydrate like lactose/ glucose/ sucrose and mannitol.To determine the antibacterial activity of bacteriocin, pathogenic bacteria *Klebsiella* and *E.coli* are used which showed inhibition zone against *Klebsiella* i.e. they are susceptible but no inhibition zone against *E.coli*. Bacteriocin considered as safe (GRAS) and used in antibiotic to inhibit the pathogenic growth of micro-organisms. The isolates were characterized by molecular method; 16S rRNA universal primers of 16S rRNA and 1492R were used for species confirmation. In the end, study elucidating their probiotic and beneficial properties may cover the way for commercial preparation of curd and darolac, biological preservation of food product and also biomedical application.

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