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EXTRA CELLULAR PRODUCTION OF ANTI MICROBIAL PEPTIDES FROM PROBIOTIC BACTERIA AND ITS MEDICAL APPLICATION

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ABSTRACT
The Isolation of probiotic bacteria namely *Lactobacillus plantarum* strains produces secondary metabolites, their screening and medical application in Gauze were carried out. The compound was confirmed using UV Visible spectroscopy, column chromatography, DNA sequencing, and were visualized by SDS-PAGE, characterized by estimating the relative molecular weight using Mass spectra. The compound was further purified by using ammonium sulphate precipitation and dialysis. The medical application of this compound is tested with the anti-textile activity with various pathogens.

Key word : *Lactobacillus plantarum*, Probiotic bacteria, *E.coli*, *K.pneumoniae*, *S.typhi*, Lactic acid bacteria, Antimicrobial peptides.

1. INTRODUCTION

There are different kinds of bacteria that may help us in numerous ways, probiotic bacteria are one example of such groups. Lactic acid bacteria is one of the major probiotic bacteria which are commonly present in Dairy products, such as Milk, Curd, yogurt, buttermilk, cheese, butter, etc. Lactic Acid Bacteria (LAB) are Gram positive, non- sporing, cocci shaped

bacilli or rods with a DNA base composition of less than 53 Mol % G+C content, anaerobic, catalase negative, ferment glucose primarily to lactic acid then to carbon dioxide and ethanol. The LABs are industrially important due to their fermentative ability and comprises of a diverse group of organisms united by the formation of lactic acid as the primary metabolite of sugar. These bacteria are devoid of cytochromes, non-aerobic but aero tolerant, fastidious, acid tolerant, strictly fermentative, lactic acid is the major end- product by homolactic fermentation, chemo – organo trophic, grows in complex media, non-pathogenic to human beings and animals. Several genera include: *Carnobacterium, Enterococcus*, *Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcis, Pediococcis, Streptococcus, Tetragenococcus , Vagococcus* and *Weissella* bases on similarities in physiology, metabolism and nutritional needs, these genera are grouped together.

The term probiotics is a portmanteau of two words ‘pro’ which is a Latin preposition meaning ‘for’ and ‘bios’ is a Greek word meaning is ‘life’. Therefore, the term probiotic means live microorganisms that when administered in adequate amounts, confer health benefits on the host. In addition they improve the quality of life by boosting the immune system, maintaining the balance of intestinal micro flora, increasing bioavailability of nutrients such as vitamin B, releasing active compounds which suppress the growth of potential pathogenic bacterias. (Nitin desai et al., 2020). Probiotics are used since long times as foods from the history of man consuming fermented foods that is well known from Greeks and Romans, that does not cause any side effects.

Cow milk is a pale liquid produced by the mammary glands of cow, a primary source of nutrition for infant mammals before they are able to digest other foods. It contain many essential nutrients namely protein and lactose (Pehrsson et al., 2000). Cow milk and their by-products are widely consumed in developing countries due to their therapeutic and nutritional values. Lactose mal-digestion occurs frequently in the general population and tolerance of Lactose in yogurt than that contained in the milk. (kajal alam et al., 2017). Human intestine comprises of billions of beneficial bacteria which helps in healthy digestion, maintaining gut health, boosts immunity etc. Many factors like stress conditions, diet, use of drugs, some treatments, etc can eventually reduce the concentration of these micro -floras. Therefore, the oral consumption of probiotic microorganisms through their natural sources like dairy products is required to produce a protective effect on the gut micro flora. The most commonly used probiotics are lactic acid producers like *Lactobacillus* and *Bifidobacteria.*

The use of chemical preservatives such as Nitrites and sulphur dioxide may cause adverse effects on human health and also the nutritional level may get imbalanced. Due to the traditional food preservation practices, the food quality may get affected because of the excess use of chemical preservatives bacteria can develop resistance as well. (Saeed et al., 2009). The major benefit of using antimicrobial peptides is that it preserves the food without changing the food's quality: texture, colour, flavor, etc. Food products can be preserved by using microbes and their antimicrobial products, which improve the self- life of food and enhance the food Safety. (Galvez et al., 2014).Lactobacillus plantarum is a flexible and versatile specie that is commonly found in various natural sources like dairy products, meat, many vegetables or plant fermentations, moreover, it is frequently encountered as a natural inhibitant of the human gastrointestinal tract (GI). (Daeschel et al., 1987; Hammes et al., 1990). L. plantarum is a Gram positive, non –sporulating and hetero-fermentative lactic acid bacterial species, utilizes carbohydrate sources as substrate. Rod shaped microbes, about 0.9 to 1.2mm *3 to 8mm long, occurs as single, pair or in chains. These bacterias often reduce the side effects of antibiotics and have a protective effect on the intestinal tract against the harmful effects induced by E. coli.

One of the major feature of this species is the ability to produce antimicrobial compounds, including hydrogen peroxide and some organic acids and bacteriocins. They helps to avoid the integration of harmful bacteria into the human hosts, therefore they became important for the balance of ecological relationships in environment. In particular bacteriocins have a variety of potential uses such as in vererinary medicines, Pharmaceutical applications,crop management, food preservation and medical applications. Bacteriocins are low molecular peptides secreted by the bacterial cells to kill sensitive cells present I the same ecosystem, they exhibitantimicrobial, antiviral and antifungal properties. They are found to be effective against the human skin pathogens like Candida albicans, Malassezia sps. (Aparna Ravi, et al., (2024) Bacteriocin and its effect against skin pathogens. International journal of Microbiology and Mycology. Vol.18, No.2, p.1-7, 2024.INNS PUB).The compound is isolated purified and confirmed using standard molecular tools.

2. MATERIALS AND METHODS

Collection of Bacteria

Bacterial cultures were collected from Microbial Type Culture collection Centre (MTCC).

2.2 Identification of Bacteria

2.2.1 milk Coagulation Assay

For milk coagulation test, 100% fresh one night culture of the probiotic bacteria was added into 10% sterile skim milk agar and incubate at 37 degree Celsius for 48 hours in incubator, their optical density at 610nm was determined to check the growth.

2.2.2 Bile Tolerance Test

The bile salt tolerance of the isolates was determined by incubating the isolates in 20ml of MRS broth containing 4% NaCl. After incubation at 37 degree Celsius for 24 hours, optical density at 610nm was determined to check growth.

2.2.3 Subculturing of Bacteria

20 ml of nutrient agar was prepared, sterilised at 121 degree Celsius for 15 minutes, poured into petri plates and allow it to get solidify. The plate was inoculated with loop full of bacterial culture with Quadrant streak, then the plates were incubated at 37 degree Celsius for 24 hours. After 24 hours the plates were observed for colony formation.

2.2.4 Preparation of Production Media

About 10% inoculum was added in separate flasks containing 50 ml production media for checking the secondary metabolite production. The production media contained (g/l) protease peptone (10.00), peptone (10.00), yeast extract (5.00), Polysorbate 80 (1.00), dextrose (20.00), ammonium citrate (2.00), sodium acetate (5.00), magnesium sulphate (0.10), manganese sulphate (0.05), di pottasium hydrogen phosphate (2.00). MRS broth was taken in a respective quantity for 50ml distilled water, Sterilize at 121 degree Celsius for 15 minutes.

3. Precipitation and Purification of Compound

3.1 Ammonium Sulphate Precipitation

The media was centrifuged at 5000rpm for 10 minutes. The supernatant was collected in the conical flask, Ammonium sulphate (70%) was added into the supernatant for precipitation, incubated in the refrigerator for 24 hours. Then the sample is centrifuged at 6000 rpm for 5 minutes, the resulting supernatant is used for dialysis.

3.2 Dialysis

The collected precipitated samples from the salt purification was transferred into the dialysis membrane, was incubated against phosphate buffer solution for 24 hours in the refrigerator.

3.3 Column Chromatography

The column uses Sephadex G for the purification of enzyme product. Chromatography was carried out on a DEAE-50 cellulose ion exchange column (2.5 cm* 50cm; Whatman) previously equilibrated with 0.02 mol/L phosphate buffer (pH6.8) for rinsing added tap water to the column and then eluted. Then added 10ml of precipitated enzyme (crude+ ammonium sulphate), collected and compound was purified in different Eppendoff tubes (Fractions 1, 2, 3, 4, 5, 6). Each fractions were analysed by using spectrophotometric study at 280nm.

4. Characterisation of Compound

4.1 UV Visible Specrophotometer

The secondary metabolites were confirmed by measuring the wave length of the reaction mixture in the UV- VIS Spectrum of the Elico SL 159 at a resolution of 1nm (from 300-600nm) in 2ml quartz cuvette with 1cm path length. UV- Visible study was done to identify the compounds and the diluted supernatant was analyzed in the range from 200-400nm.

4.2 Separating Gel

Gel was prepared by mixing 4ml of dist. water, 3,3ml of acrylamide, 2.5 ml of 1.2 M tris buffer, 0.008ml of TEMED, 0.1 ml of APS. APS was used to solidify.

4.3 Stocking Gel

Mixed 3.4ml of dist. water, 0.830 ml of acrylamide, 0.630ml of 1M tris buffer, 0.05ml of 10% SDS, 0.05ml of TEMED, 0.05ml of APS.

5. SDS-Page

Prepare 10% resolving gel as well as preparation of stacking gel and running buffer should be prepared. Setup gel electrophoresis and pour the running gel place 1ml of Dist. water saturated with butanol carefully on the top of monomer solution and keep for 50 min. To allow the gel to polymerize add the stacking gel buffer with APS and TEMED being added just before pouring insert sample comb to allow the air bubble escape keep set for 20 mins and then remove the comb, place the gel in the buffer chamber and add running buffer to cover the gel.

30ML of the sample collected from column chromatography was taken in a PCR tube. 20ml of SDS loading dye was added to it, mixed well and incubated at 90 degree celsius for 15-20 mins. After heating load the sample into the SDS-PAGE.

5.1 Staining Dye Preparation

Take 9ml of water, 9ml of methanol, 2 ml of acetic acid, mixed with 0.5% of coomassive brilliant blue.

5.2 Destaining Dye Preparation

Take 9ml of water, 9ml of methanol and 2ml of acetic acid, mixed well. The gel from SDS-PAGE was placed into the staining dye and allowed for overnight incubation, the gel was placed in the de staining dye and allowed for overnight incubation.

6. Medical Applications

6.1 Turbidity Assay

20ml of nutrient broth was prepared and sterilized at 121 degree Celsius in 15lbs pressure. 8 sterile widal tubes were filled with 2ml of cooled broth, divide the widal tubes into two sections; one is crude sample (each marked with C50ml, C100ml, C150ml, C 200ml) and other is purified sample (each marked with P50ml, P100ml, P150ml, P200ml). Added the purified and crude sample into respective widal tubes, then added 20 ml of *E.coli* sample into all the tubes and mixed well, incubated at 37 degree Celsius for 24 hours.

6.2 Anti Textile Activity Using Gauze Cloth

The test was carried out in the Muller Hinton Agar medium. Media was prepared for100ml of dist. Water, after sterilization the madia was pouredinto 3 plates forsolidification, was swabbed with *E.coli, S.typhi,* and *K.pneumoniae* cultures respectively. Place the gauze cloth cotton coated with column sample and crude sample. The disc Methycillin (MET) was placed in the plate was positive control, incubated the plates at 37 degree Celsius for 24 hours, Zone formation was observed and the diameter of zone of clearance was measured. (Aparnaravi et al., 2024).

3. RESULTS

1. Identification and conformation of bacteria

1.1 Milk coagulation assay

The Milk coagulation assay was carried out for the determination of the growth of bacteria. The optical density was measured at 610nm and was found to be 0.36nm and 0.44nm (Figure 1, Table 1).

Table -1 Milk coagulation assay

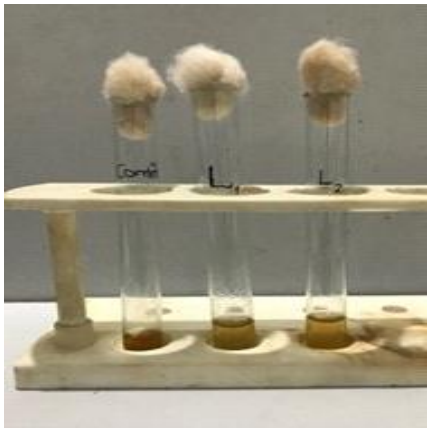
S. No	Samples	OD value at 610nm
1.	Control	0.00
2.	Skim milk 1	0.36
3.	Skim milk 2	0.44

1.2. Bile tolerance test

The bile tolerance test was carried out for the determination of bile tolerance of bacteria. The optical density was measured at 610nm and was found to be 0.79nm and 0.33nm (Figure 2, Table2).

Table-2 Bile tolerance test

S. No	Samples	OD value at 610nm
1.	Control	0.00
2.	MRS broth + culture	0.79
3.	MRS broth + culture + NaCl	0.33



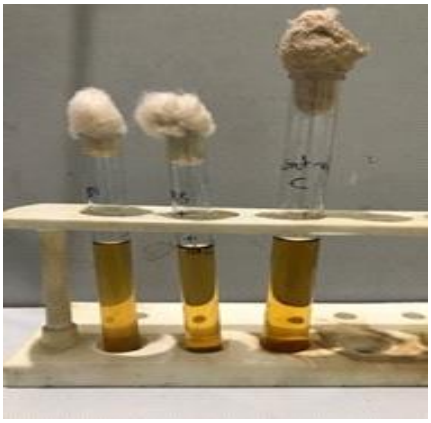


Figure -1 Milk Coagulation Assay **Figure-2 Bile Tolerance Test**

2. Subculturing of bacteria

The bacteria was sub cultured and used for further studies. Further studies will be done on the characterization, identification in to species level and their probiotic potential (Figure 3).

3. Production of secondary metabolites

The secondary metabolites are produced by inoculating *Lactobacillus plantarum* species on production media (Figure 4).

4. Precipitation and purification of compound

4.1. Ammonium sulphate precipitation

Thus the secondary metabolites are purified by using 70% of ammonium sulphate by the method of salt precipitation (Figure 5).

4.2. Dialysis

The dialysis was carried out for the production of secondary metabolites. The optical density was measured at 280nm and was found to be 0.128nm (Figure 6).

Table-3 Dialysis

S. No	Samples	OD value at 610nm
1.	PBS (Phosphate buffer solution)	0.00
2.	Sample 1	0.128

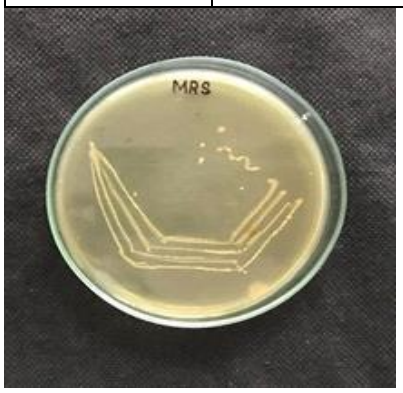


Figure 3– Subculturing of Bacteria. **Figure 4 – Production of Secondary Metabolites**

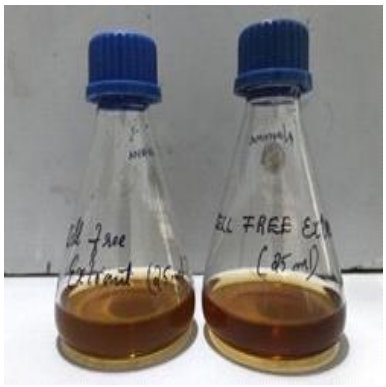


Figure-5 Ammonium Sulphate Precipitation **Figure-6 Dialysis**

4.3. Column chromatography

The secondary metabolites was purified by using column chromatography using Sephadex G-50. Among the 6 fractions, collected fraction 4 have maximum activity (Figure 7).

Table-4 column chromatography.

Fractions	**OD value at 280 nm**
Blank	0.00
1	0.078
2	0.122
3	0.114
4	0.347
5	0.275
6	0.103

5. Characterisation of compound

5.1. UV visible spectrophotometer

The characterization of surface protein extracted from *Lactobacillus plantarum* was monitored by UV – Visible spectrophotometer analysis. The dilute supernatant was analysed between 200 – 400 nm wavelengths and was found to be 0.347 maximum.

6. SDS PAGE

The sample proteins are separated by sodium dodecyl sulphate-poly acrylamide gel electrophoresis. The proteins appeared as discrete bands in the gels. The relative molecular weights of the protein with respect to their bands were observed in 28 kDa(kilo Daltons) and 39 kDa (Figure 8).

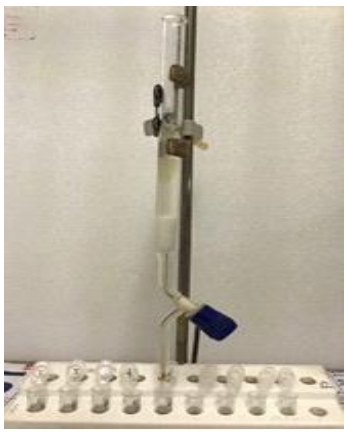


Figure-7 Column Chromatography.

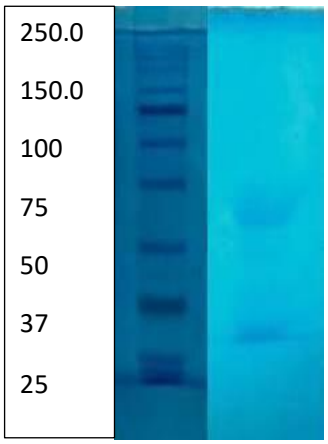


Figure-8 SDS PAGE

4.7. Medical applications

4.7.1 Turbidity assay

Turbidity was against *E. coli* was also studied and the maximum activity was found to be 56.87%.

Percentage of cell death = [Control OD – Sample OD /Control OD] × 100

1. Crude sample

(Death %) sample C50 = (1.036– 0.80/1.036)100 =76.18 %

(Death %) sample C100 = (1.036-0.60 / 1.036) 100 =56.87 %

(Death %) sample C150 = (1.036-0.56 / 1.036) 100 =53.01 %

(Death %) sample C200 = (1.036-0.60 / 1.036) 100 =56.87 %

2. Purified sample

(Death %) sample P50 = (1.036-0.75 / 1.036) 100 =71.35 %

(Death %) sample P100 = (1.036-0.56 / 1.036) 100 =53.01 %

(Death %) sample P150 = (1.036-0.49 / 1.036) 100 =46.26 %

(Death %) sample P200 = (1.036-0.40 / 1.036) 100 =37.57 %

Table-5 Turbidity Assay- I

S. No	Crude sample (C)	OD value at 600nm	Percentage(%) of cell death (control- 1.036)
1	C50	0.80	76.18%
2	C100	0.60	56.87%
3	C150	0.56	53.01%
4	C200	0.60	56.87%

Table-6 Turbidity Assay - II

S. No	Crude sample (C)	OD value at 600nm	Percentage(%) of cell death (control- 1.036)
1	P50	0.75	71.35%
2	P100	0.56	53.01%
3	P150	0.49	46.26%
4	P200	0.40	37.57%

7.2 Anti textile activity using gauge cloth

The antibacterial activity was estimated and found to be maximum against *S. typhi*(5- 9nm) followed by *E. coli*(5nm) *and K. pneumoniae*(2nm) (Figure 9).

Table-7 Anti textile activity using gauge cloth

S. No	Organism Name	Zone of inhibition		
		Crude	purified	Disc
1.	*E. coli*	2nm	9nm	Nil
2.	*S. typhi*	5nm	9nm	Nil

3.	*K .pneumoniae*	2nm	9nm	Nil

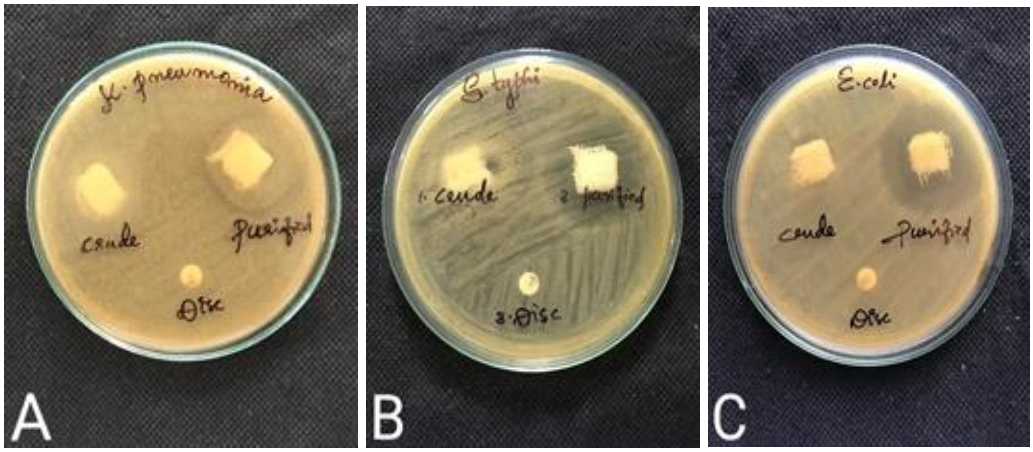


Figure-9 Anti textile activity using gauge cloth a)*K. pneumonia b)S.typhi c)E.coli*

DISCUSSION

The extracellular synthesis of antimicrobial peptides from probiotic bacteria (Lactobacillus plantarum MTCC 1325) and its potential medical uses were the aim of the current study. The Lactobacillus plantarum strains use whole, complete cells as their target in order to create secondary metabolites. With the use of column chromatography and UV-visible research, the substance was further verified. Using mass spectra to estimate the relative molecular weight, the secondary metabolites are characterized and visualized using SDS-PAGE. The chemical underwent dialysis and ammonium sulphate precipitation for further purification. Several microorganisms were used to test the antibacterial activity.

The primary purpose of MRS agar was to cultivate lactobacilli from different sources and provide a standardized medium that could replace tomato juice agar. The media is specific for growing members of the lactic acid bacteria.It turns lactic acid bacteria selective by dropping the pH to 5.7 and adding 0.14 percent sorbic acid. MRS agar provides different colony size and morphology in addition to high colony counts for lactobacilli and other lactic acid bacteria. Testing for the relevant colonial presence and confirmation eliminates other bacteria.

The presumed LAB isolates were subsequently identified and confirmed by bile tolerances and the milk coagulation test in order to verify viability under gastrointestinal pH

circumstances. The ability of a probiotic strain to withstand bile salts, pancreatic fluid, and stomach pH is essential for forecasting its survival and growth in gastrointestinal disorders. One of the main functional requirements for probiotics to survive during transit through the gastrointestinal tract is their ability to withstand gastric acidity (pH 2.0–2.5).

Studies using 0.3% Ox gall dissolved in MRS broth to test the isolates' bile tolerance yielded results that were equivalent to those of sodium taurocholate, a significant component of bile in carnivorous mammals, including humans. Although all LAB isolates remained viable over a 4-hour incubation period in the presence of 0.3% ox gall, isolates 4, 13, 32, and 36 demonstrated a survival rate exceeding 50%, a threshold deemed acceptable for a prospective probiotic strain. This suggests that intestine origin isolates had a better survival rate when added to media enriched with ox gall. Probiotic strain candidates ought to be able to withstand a pH of at least 3.0, if not lower. An abundant source of LAB is raw milk. Furthermore, the presence of both homofermentative and heterofermentative lactic acid bacteria in raw cow milk was demonstrated.

Every stage of the purification process led to a significant drop in protein concentration while increasing specific activity. The inclusion of ammonium sulphate and trichloroacetic acid precipitations resulted in the best bacteriocin recovery. 70% ammonium sulphate was used in the salt precipitation procedure to purify the secondary metabolites. The dialysis was carried out for the production of secondary metabolites. The optical density was measured at 280nm and was found to be 0.128nm. The secondary metabolites were purified by using column chromatography using Sephadex G-50. Among the 6 fractions, collected fraction 4 have maximum activity.

A combination of morphological and genotypic approaches was used to group strains before stress tolerance was evaluated, the confirmation of identification at the species level and finger printing of 63 strains of L. plantarum. Previous methods of identification for the majority of bacteria used partial sequencing of the 16 S rRNA genes or phenotypic techniques such as whole cell protein SDS-PAGE. Due to their close genetic relationship, L. plantarum, L. pentosus, and L. paraplantarum, each of these methods may only be partially successful in identifying the species. In fact, SDS-PAGE of whole cell proteins was only able to discriminate *L. pentosus* from *L. paraplantarum* and *L. plantarum.* (Teresa *et al*.,2010). The sample proteins are separated by sodium dodecyl sulphate-poly acrylamide gel electrophoresis. The relative

molecular weights of the protein with respect to their bands were observed in 28 kDa and 39 kDa.

The probiotic bacteria's ability to block pathogenic organisms is one of its most significant features. LAB are capable of producing significant amounts of organic acids, which hinder the growth of harmful bacteria. Furthermore, a small number of isolates also generate the antibacterial compound bacteriocins. By producing these substances, LAB is able to drive out the harmful bacteria through competition. Each isolation had a different level of hostility. Among all the test organisms, isolate 36 (L. plantarum) exhibited the strongest antagonistic properties. When compared to the market isolate, the majority of the isolates exhibited antagonistic activity that was either higher or equal. When compared to the market strain isolate, all of the isolates shown greater antibacterial activity against S. aureus. The highest activity of turbidity against Escherichia coli was thus shown to be 60%.

This suggests that the diffusible and extracellular inhibitory metabolites generated by isolated Lactobacillus species were present. The lactobacilli found in curd, a classic fermented milk product, are resistant to inhibitors and can thrive in both acidic and alkaline environments. They exhibited antibacterial activity against some indicator pathogens. Based on these characteristics the isolates may have potential for natural preservatives and may also be considered for probiotic application. (Somnath *et al*.,2017). Thus the antibacterial activity was estimated and found to be maximum against *S. typhi*(5- 9nm) followed by *E. coli*(5nm) and *K. pneumoniae*(2nm).

CONCLUSION

This study examined the anti-textile effects of partly pure Lactobacillus species secondary metabolites using column chromatography. Following E. coli and K. pneumoniae, the secondary metabolites demonstrated antibacterial efficacy against S. typhi (5–9 nm). The anti-textile property showed the highest action against S. typhi (9 nm), E. coli (5 nm), and K. pneumoniae (2 nm). The current investigation came to the conclusion that the secondary

metabolites that were separated might be employed as an antibacterial agent to combat a variety of harmful bacteria.

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Nil

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