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DETECTION OF ANTAGONISTIC ACTIVITY OF BACTERIOCIN PRODUCING BACTERIA FROM YOGHURT

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ABSTRACT

Bacteriocin, are considered as potent antimicrobial agent against multi drug-resistant (MDR) pathogen. Using selective media, *Lactobacillus spp*. was isolated from commercially available yogurt. *Lactobacillus spp* were isolated using De Man, Rogosa, and Sharpe (MRS) agar media. Various types of bacteriocins have been chemically, biochemically, and genetically described. Gram staining was used to identify the morphology of the *Lactobacillus spp*. Catalase, oxidase, MRVP, and indole test have all been used to identify it biochemically. The antibacterial activities of *Lactobacillus spp.* against the host bacterium *P. aeruginosa* were confirmed using the agar well diffusion method. The result of antimicrobial activity was measured by the arbitrary unit (AU/ml) of the zone of inhibition. Bacteriocin protein minimizes the usage of chemical preservatives and may be used as a bio-preservative in the food sector in the future. In food industry alternative to chemical preservatives bacteriocin is used as a natural preservative, nisin is the only bacteriocin that has been officially approved as a food preservative by regulatory agencies. Bacteriocins, with their broad or narrow spectrum effectiveness against antibiotic-resistant bacteria, could be a potential solution to this global problem.

Keywords: *Lactobacillus spp*, Bacteriocin, Nisin, Antagonistic activity, Bio-preservation

1. INTRODUCTION

Bacteriocins are antibacterial proteins or short polypeptides produced by gram positive bacteria. Bacteriocins interact with and kill cells that have specific surface receptors. A total number of 3 bacteriocin cells exist in the microbial community i.e., bacteriocin genic cells (those that produce bacteriocin), sensitive cells (sensitive to bacteriocin), and resistant cells (resistant to bacteriocin) **[1]**. Because of their physiologically active nature, they are considered as a medicinal drug which act against several strains of bacteria. They are produced by bacteria and are not commonly referred to as antibiotics which might have side effects. The main distinction between bacteriocin and antibiotics is that bacteriocin only acts on related species of bacteria and specific strains of those species. On the other hand, antibiotics have a huge spectrum of activity **[2]**. Andre Gratia discovered the first bacteriocin in 1925, he noticed that a strain of verotoxin producing *Escherichia coli* V stop the production of other strains of *E. coli* **[3]**. There is a variety of bacteriocins present in the environment, with modifications in shape and process of action. Numerous research studies revealed that bacteriocins are mostly produced by Gram-negative bacteria such as *Pseudomonas sp. Salmonella sp. Yersinia sp. Aeromonas sp., and Escherichia sp.* **[4, 5]**.

Table 1: Bacterial Genera that produce Bacteriocins

Haemophilus	***Acetobacter***	***Salmonella***
Haloferax	***Actinobacillus***	***Propionibacterium***
Lactobacillus	***Bacillus***	***Serratia***
Lactococcus	***Brevibacterium***	***Shigella***
Listeria	***Clostridium***	***Staphylococcus***
Leuconostoc	***Corynebacterium***	***Streptococcus***
Pseudomonas	***Enterococcus***	***Yersinia***
Pediococcus	***Erwinia***	

The only FDA approved bacteriocin is NISIN (Group N Streptococcus Inhibitory Substance IN) is an antimicrobic peptide that is produced by Gram-positive bacteria such as *Lactococcus* and *Streptococcus*. Nisin was discovered in fermented milk cultures in 1928 and was first commercially sold as an antibacterial agent in England in 1953. Nisin has antibacterial action in contrast to a variety of Gram-negative bacteria, with a special affinity for microbial spores **[6]**. The Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) validated nisin as a safe food ingredient in 1969. Nisin is currently approved in more than 50 countries and has had a considerable impact on the food sector as a natural bio preservative for various foods **[7]**. The Food and Drug Administration approved nisin for use in packaged cheeses in the United States (US) in 1988, and it was generally regarded as safe certification **[8]**.

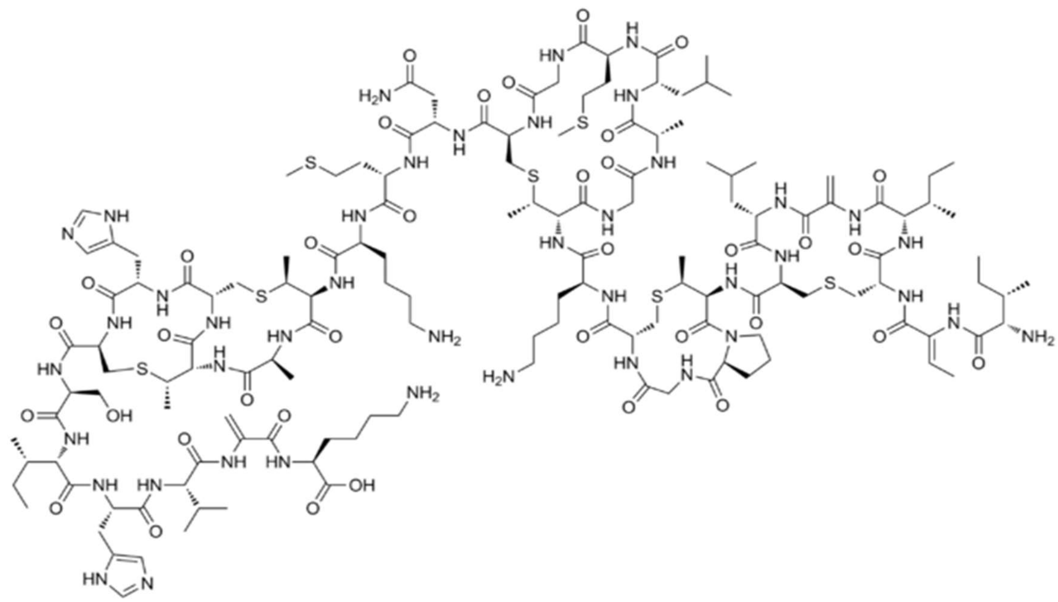

Figure 1: Primary structure of Nisin

Bacteriocins are grouped depending on the molecular size, thermostability, enzymatic sensitivity, method of action, and presence of post-translationally altered amino acids **[9]**. Jack *et. al* 1995 reported that disulfide and monosulfide (lanthionine) bonds impact the activity range of bacteriocins **[10]**. Gram-negative bacteriocin are separated into 2 types based on their molecular weight i.e., colicins and microcins **[11]**. Gram-positive bacteriocins were classified into three groups based on biochemical and genetic features. It was reported by various scientist's that gram positive bacteriocin are further classified into two and three subclasses in classes I and II, respectively **[12]**. Bacteriocin categorization has been evaluated regularly as per the latest classification, based on their structural and physiochemical features, Gram-positive bacteriocins are now classified into 3 main groups **[13]**.

Archaea produce a unique family of antimicrobial peptides called archaeocins that are similar to bacteriocins. Researchers have found that till date 2 types of archaeocins have been discovered; halocins and sulfolobicins **[14-16]**. Halocins are further categorized based on their size. The smaller microhalocins consist size of 3.4 kDa, whereas the bigger one have a size of 35 kDa. In general, they affect cell permeability or stop the sodium/hydrogen antiporter and proton flux that acts on the cell membrane **[17]**. Sulfolobicins are created by the *Crenarchaeota phylum's, Sulfolobus islandicus.* This bacterium thrives at a pH of 2-4 at a temperature of

65–85°F. Sulfolobicins are bacteriocins with a narrow spectrum of activity that suppress the growth of Sulfolobus and its associates. The mechanism of action of these bacteriocins is unknown **[16-18]**.

Table 2: Classification of Bacteriocins

BACTERIA NAME	CLASS	SIZE (KDA)	EXAMPLES
Gram-negative bacteria	*Colicins*	30-100	Colicins A, B, E2, E3
	Colicins-like bacteriocins	30-100	S-piocins, Klebicins
	Phage tail-like bacteriocins	20-100	R- and F-piocins
	Microcins	<10	Microcin C7, Microcin B17, Colicin V
Gram-positive Bacteria	*Class I*	<5	Nisin, Mersadin, Lacticin 3147
	Class III	<10	Pediocin RA1, Carnobacteriocin B2
	Class III	>10	Helvecin, Enterocin AS-48
ARCHEA	Halocins	>5	Halocin A4, C8, H1, H4
	Sulfolobacease	~10	Sulfolobacease

Microbe isolation as well as screening from natural sources has traditionally been the most effective method for obtaining beneficial and genetically reliable strains for industrially important products.

In this study, we have identified and isolated bacteriocins from lactic acid bacteria that could be useful in bio-preservation. Isolated bacteriocin were further tested for its Gram's nature as well as its biochemical test. LAB from yoghurts were tested further for its antimicrobial activity on host bacteria *P. aeruginosa* and the zone of inhibition was measured.

2. MATERIALS AND METHODS

2.1. Isolation of Lactic Acid Bacteria (LAB).

De Man, Rogosa, Sharpe *Lactobacilli* (MRS), and agar were used to culture lactic acid bacteria (LAB) when originally isolated from commercially available Amul yogurt, incubated at 37° C for 40-70 hrs. The colonies were picked up and obtained after incubation and re-streaked onto MRS medium to obtain pure isolates.

2.2. Identification of *Lactobacilli sp.*

Lactobacillus spp. was characterized by the step-by-step procedure described below.

Microscope observation.

The isolated strains were observed under a microscope for its structure, Gram's nature was identified after doing Gram's staining procedure, at last motility test was done using hanging drop method.

2.3. Biochemical Test

The following biochemical test was performed for the identification of *Lactobacillus sp.*

1) Catalase Test

On the glass slide, place one drop of 3 % hydrogen peroxide (H2O2). A sterile loop was used to pick up the colony and place it in the three percent hydrogen peroxide mixture. Wait for the bubble to appear after 2 minutes.

2) Oxidase Test

A culture of bacteria was grown in 10ml of nutrient broth. Few drops of oxidase reagents were mixed in broth and were shaken forcefully and were observed for color changes within 10-40 seconds.

3) Methyl-Red Test

This test is used to detect the organism which produces acid during the fermentation of glucose. The ingredients were dissolved in D/W and the medium was dispersed in 10 mL volumes in test tubes after the pH was adjusted to 7.5. Solution was autoclaved. The plates were inoculated with the specific/test organisms and incubated for 48-72 hours at 37°C with 5 drops of methyl red indicator (0.1g of methyl red in 300 mL of 95 percent ethanol, made up to 500 mL in distilled water). Few drops of the methyl-red indicator were added to see the red color formation.

4) Voges Proskauer

1-2 colonies were added to the medium and incubated in the incubator. Barritt's reagent A and Barritt's reagent B were added to the medium and were shaken for a few minutes and pink-red color was observed if formed.

5) Indole Test

10ml of tryptophan broth was taken in tubes. Strains were added from fresh growth and were incubated at room temperature for 16-23 hrs. 1-2ml of Kovac's reagent was added to the culture, and the presence or absence of a ring was observed.

2.3. Production of Bacteriocin

Bacteriocin is a secondary product formed by the strain of LAB bacteria. For the production of bacteriocin, fresh colonies were inoculated in fresh MRS broth, the broth was incubated within a shaking incubator at room temperature for 36-70 hrs. After the incubation period turbidity was shown in the broth.

2.4. Extraction of crude Bacteriocin

A minor alteration was used to extract the bacteriocin. Selected Lactobacillus isolates were cultivated in MRS broth for forty hours at 130 rpm in a shaking incubator. The organic acid's antibacterial effect was then removed by centrifuging the cultures at 8000 g for 20 minutes at 4 °C and adjusting the pH to 7.0 with 1M NaOH. The isolated bacteriocin was stored at 4°C.

2.5. Screening of Isolates for Antimicrobial Activity

Well Diffusion Method

Using the well diffusion method, the isolated bacteriocin's antibacterial properties were ascertained. 200 µl of samples were put into 6 mm-diameter wells cut out of agar plates. On nutrient agar, the inhibitory action against Pseudomonas aeruginosa was developed. Plates were incubated at 37 ° c for 16–24 hours after being held at a cold temperature for 1.5 hours. The diameter of the inhibitory zone surrounding the wells was measured to determine the antibacterial activity **[19]**.

3. RESULTS

3.1. Isolation & identification of lactic acid bacteria

By examining the colony shape, physiological, and biochemical features of the bacteria isolated from Amul yoghurt, *Lactobacillus spp.* were recognised. On the MRS agar plate, each isolate displayed white, tiny sticks, rounded colonies, and creamy color.

3.1.1. Microscopic observation

The isolated colony was screened using Grams staining and then the slide was seen under a microscope at 100x to see the shape and Grams's nature. Microscopically isolates showed Gram-positive, rod-shaped bacteria shown in **Figure 3**.

3.1.2. Biochemical Tests

Below are the results of the biochemical tests **(Table 3)**.

3.2. Production of bacteriocin

Fresh colonies were added to MRS broth, which was then incubated for 36–72 hours at room temperature in a shaking incubator. In broth, turbidity was observed **(Figure 4)**.

3.3. Extraction of crude bacteriocin

The crude bacteriocin was extracted by performing centrifugation as described in the materials & method.

3.4. Anti-microbial activity of lactic acid bacteria by agar well diffusion

The mature grown colonies were kept for further incubation. After incubation of 48-72 hrs., the crude bacteriocin was extracted by centrifugation. Few drops of extracted bacteriocin sample were kept by agar well diffusion method in host bacteria of *P. aeruginosa*. The zone of inhibition was shown after 24 hrs. of incubation. The results of antimicrobial activity of *Lactobacillus spp.* against the test organisms revealed a 25mm zone of inhibition **(Figure 5)**.

Figure 2: *Lactobacillus spp*. on MRS agar media

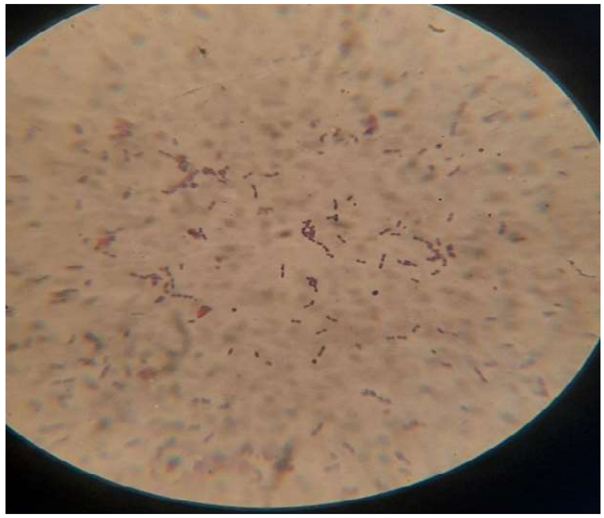

Figure 3: Gram-Positive *Lactobacillus sp.*

Table 3: Biochemical Tests

Biochemical Test	Result
Catalase	Negative
Oxidase	Negative
Indole	Negative
Methyl-Red	Negative
Voges-Proskauer	Negative

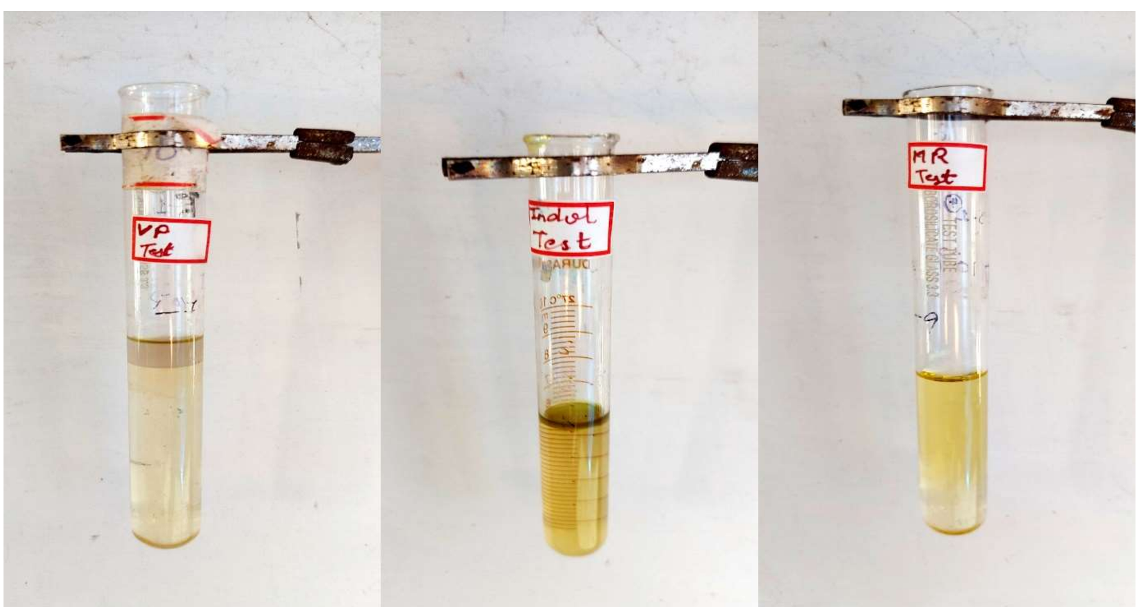

Figure 4: Various Biochemical tests

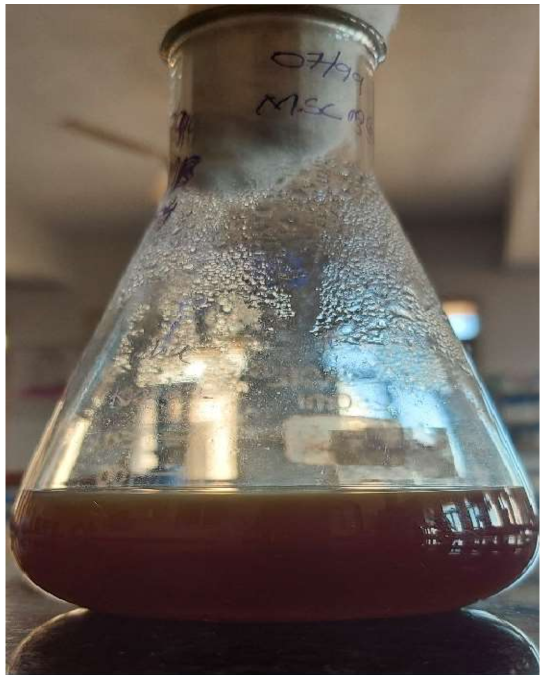

Figure 4: Turbidity of colonies

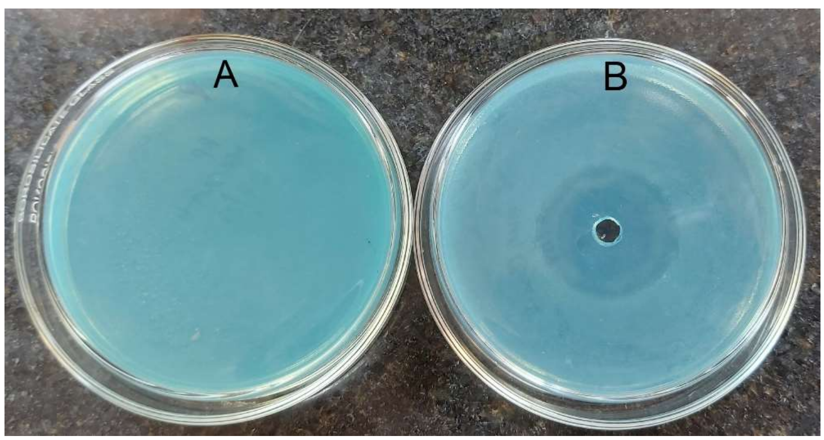

Figure 5: (A) Control, (B) Antibacterial Activity of *Lactobacillus spp.* against *P. aeruginosa*

3.5. DISCUSSION

For LAB, MRS was used as the medium, which provided a broad overview of the flora contained in the sample. Bacteriocins had a 20% success rate against *L. innocua*, and 16S rRNA gene analysis of these isolates revealed that they belonged to four different LAB species. Sharpe (2009) showed 8.7% of the 92 LAB recovered from fresh-cut vegetables contained bacteriocin-producing bacteria **[20]**, however Sezer and Güven (2009) screened 12,700 LAB isolates from milk and meat products and only identified 35 bacteriocin-producing strains **[21]**. As a result, selecting the right food source and media is critical for successfully isolating bacteriocinogenic LAB.

Islam R and his co-worker using selective media, *Lactobacillus spp.* was isolated from typical winter fermented vegetables such as cucumber and carrot. It was discovered that the isolate was facultatively anaerobic, Gram-positive, and catalase-negative. The antibacterial activity of the zone of inhibition is measured by the Arbitrary Unit (AU/ml). Six isolates were discovered in the sample, although isolate 4 showed the strongest activity against the *Bacillus megaterium* (55 mm) zone of diameter. The molecular weight of washed bacteriocin was approximated to be around 40 kDa (Isolate 1) and 15 kDa and 30 kDa (Isolate 2, 5) **[22]**.

Our study showed that the bacteria isolated from commercially available Amul yogurt were *lactobacillus spp.*

The characteristics were obtained by doing various biochemical tests and Grams nature was obtained by Grams staining. The isolated bacteriocin was tested for its antimicrobial activity against host bacteria *P. aeruginosa* and the zone of inhibition was measured 25mm.

3.6. CONCLUSION

The strains were isolated from commercially available yogurt using basic protocols. Following further research, the *lactobacilli. spp* were allowed to grow and form secondary metabolites, producing bacteriocin. The detection of BLIS was done on the indicator bacteria which results in the inhibition of the growth of host bacteria. Bacteriocin has also shown a zone of inhibition. In the future, it is predicted that bacteriocin will be employed as a therapeutic potential against multi-drug resistant bacteria, which will not only slow the spread of superbugs but may also replace antibiotics. Furthermore, bacteriocin would be used as a bio-preservative in the preservation of food & dairy products.

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