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ISOLATION AND CHARACTERIZATION OF MICROBES PRODUCING BACTERIOCIN FROM CURD, RAW MILK AND SOIL AND ITS PRESERVATIVE EFFECTS

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ABSTRACT: Bacteriocins are proteinaceous substance produced by bacteria to inhibit the growth of similar or closely related bacterial strains. They are typically considered to be narrow spectrum antibiotics produced by both gram negative and gram positive range of bacteria which possess antimicrobial properties and inhibitory towards sensitive strains. Bacterocins are safer than antibiotic. They can be used from natural material (probiotics). Bacteriocins of lactic acid bacteria are considered as safe natural preservatives or bio preservatives. A total of nineteen colonies were picked from the various samples i.e. Curd, Condensed milk, Soil and Raw milk. All were subjected to bacteriocin assay and a total of six (6) strains showing relative positive results were selected and subjected for further characterization. Among all six (6) isolates (P1, R3, R6, R7, R8, T7), P1and R7 strains showed promising results against various test organisms with the observed bacteriocin activity. During preservative studies, addition of cell free bacteriocin extract of P1 strain showed positive result for preservation of raw milk up to 4days without any change in its pH and colour with respect to other strains. The best bacteriocin producing microbe, strain P1 was identified as *Paenibacillus lactis* which is an ideal strain that can be use further in probiotics studies for higher yielding and enhancement.

INTRODUCTION: Bacteriocins are ribosomally-synthesized antibacterial peptides. These compounds are produced by a broad variety of different bacteria belonging mainly to the genus *Bifidobacterium*, to which health promoting properties have frequently been attributed.

However, despite the fact that the identification of *Bifidobacterium*-associated bacteriocins was first reported in 1980 and that they exhibit antimicrobial activity against pathogenic microorganisms such as *Listeria monocytogenes*, *Clostridium perfringens*, and *Escherichia coli*, relatively little information is still available about the antimicrobial compounds produced by strains of this genus [1].

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Bacteriocins play very important role in food preservation processes by inhibiting or killing of spoilage and/or pathogenic microorganisms in food and dairy products. The bacteriocins produced by *L. bulgaricus* and *L. lactis* were identified as bulgarican and nisin respectively. The bulgarican and nisin produced inhibited spoilage pathogenic microorganisms: *Staphylococcus*, *Salmonella*, *Bacillus*, *Shigella* and *Pseudomonas* [2].

The structure of bacteriocins consists of bacterial peptides with specific activity against competing species in addition to carbohydrate and/or lipid moieties [3]. Bacteriocins bind specifically to receptors on the surface of target cells and kill the cells by alteration of membrane-bound enzymes, disruption of membrane potential by pore formation, or by enzymatic digestion of RNA and/or DNA. The proteinaceous nature of these antimicrobial molecules as well as their natural occurrence in nature has allowed their use in foods to prevent microbial food borne diseases and

bacterial food spoilage [4]. Recently, it was reported that Lactobacillus species can produce antimicrobial substances which can be assimilated to bacteriocins and are active against Micrococcus luteus [5]. Bacteriocin is a biopreservative agent potential of suppressing growth of some contaminant bacteria in food industry but its commercial availability is limited and costly [6]. In view of the above facts, this study was initiated to isolate bacteriocin producing microbes from dairy products along with characterization and optimization of preservations of dairy product.

MATERIAL AND METHODS: Sample1-Curd, Sample 2-Condensed milk, Sample 3, 4 & 5-Soil sample (from Dwarka Sector 10, Faridabad and Hospital waste), and Sample 6-Raw milk were collected from different sources in Delhi, India. The samples were collected in sterile containers and transported to the laboratory of Helix Biogenesis, NOIDA for analysis within 24 hours.

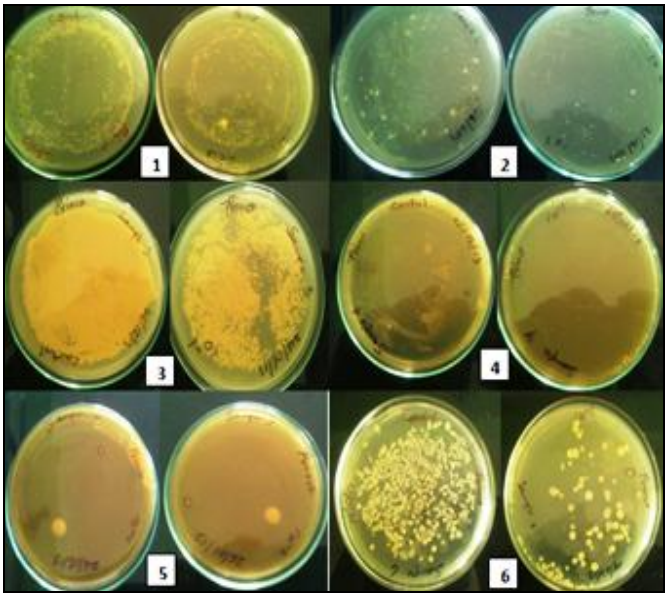


FIG. 1: SPREAD PLATE CULTURES OF SERIALLY OF DIFFERENT MORPHOLOGICAL COLONIES

Bacteriocin Assay:

Preparation of a Cell free Culture Supernatant

All the isolated strains were tested for bacteriocin production by propagating in MRS broth (pH 5.5) for 72 hours at 30°C in an anaerobic jar. Cell free solutions were obtained by centrifuging the broth culture at 3000 rpm for 15 minutes and the supernatant decanted into a new eppendorf tube

Isolation Procedure LAB was isolated according to methods of Adnan and Tanwith slight modifications [7]. Ten grams each of soil samples were serially diluted in 100ml of sterile distilled water and homogenized while 10ml of curd and milk samples were serially diluted in 90ml of sterile distil water. Serial dilution was carried out on respective samples to obtain dilution factor of 10^{-6}. 0.1 ml of appropriate serial dilution were plated with molten MRS agar plates and incubated anaerobically at 30˚C for 48 hours.

After incubation, plates were observed for bacterial growth and distinct colonies as shown in **Figure 1** and were randomly selected. Selected colonies were picked and repeatedly streaked on MRS agar plates until pure cultures were obtained. The pure cultures were maintained on MRS agar plate at 5°C after visible growth on the plate. The colony on the MRS agar plates were subcultured at two weekly intervals shown in **Figure 2**.

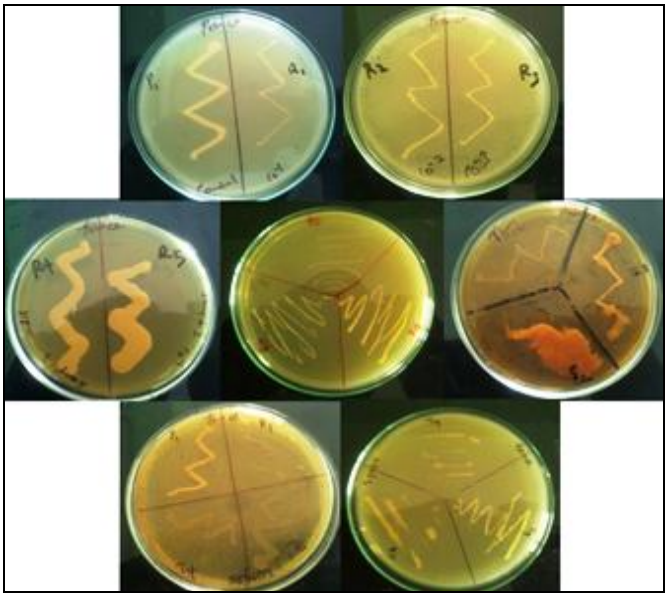


FIG. 2: STREAK PLATE NINETEEN PURE CULTURES DILUTED SAMPLES

followed by another centrifugation until a pure supernatant was gotten.

Indicator Organisms: The indicator organisms (*Staphylococcus aureus, Escherichia coli, Pseudomonas, Bacillus, L. bulgaricus, L. fermentum, M. luteus* were obtained from the Culture Collection of IMTECH. They were maintained on nutrient agar slants at 5°C and transferred at two weekly intervals.

Detection of Antagonistic Activity: The modified methods of Girum were used to determine the antibacterial activities of the isolates [8]. 0.1 ml of the broth culture of each indicator organisms were spread on already set nutrient agar plates. Wells of 5mm in diameter were cut in the agar using sterile cork borer.

A sterile pipette was used to introduce 50 micro liter of the cell free supernatant, which they were incubated for 24 hours. The plates were then checked for possible clear zones of inhibition as appeared in **Figure 3, 4, 5, 6 and Figure 7.**

FIG. 3: ZONE OF INHIBITION APPEARED IN P1 AGAINST TEST ORGANISMS 1, 2, 3, AND 4

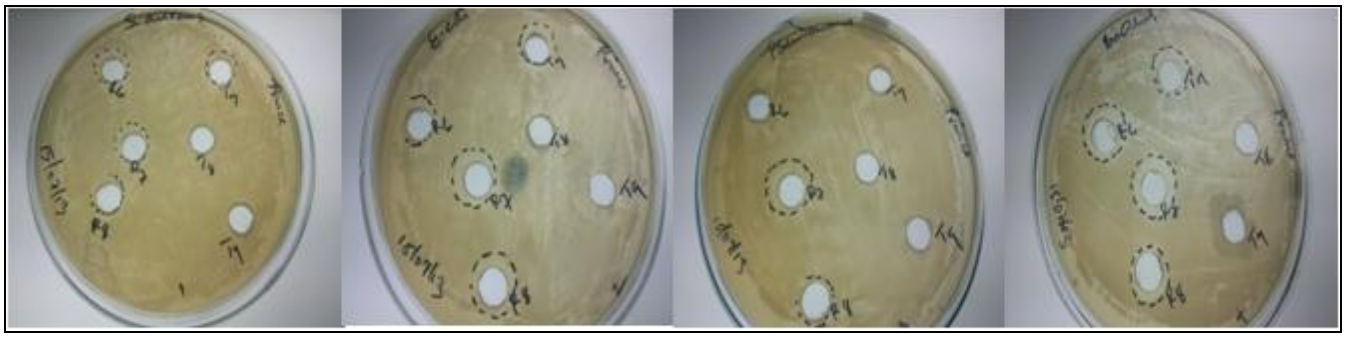


FIG. 4: ZONE OF INHIBITION APPEARED IN R3 AGAINST TEST ORGANISMS 1, 2, 3, AND 4

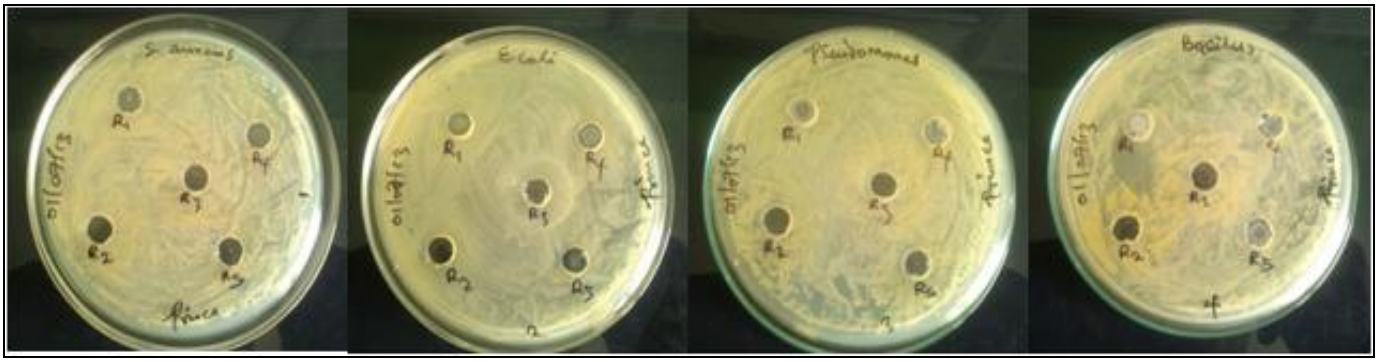


FIG. 5: ZONE OF INHIBITION APPEARED IN P1 AND R3 AGAINST TEST ORGANISMS 1, 2, 3, AND 4

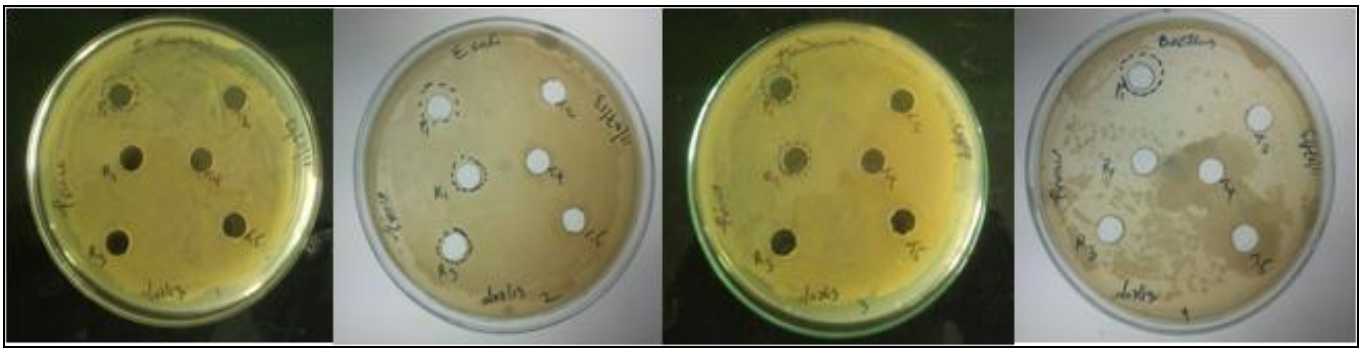


FIG. 6: ZONE OF INHIBITION APPEARED IN R6, R7, R8 AND T7 AGAINST TEST ORGANISMS 1, 2, 3 AND 4

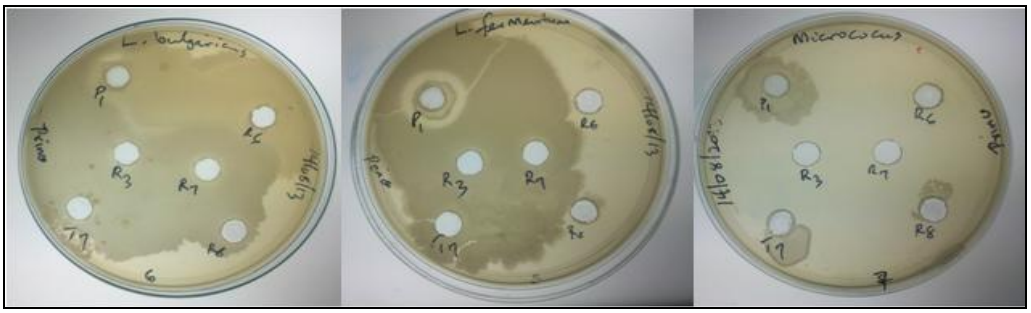
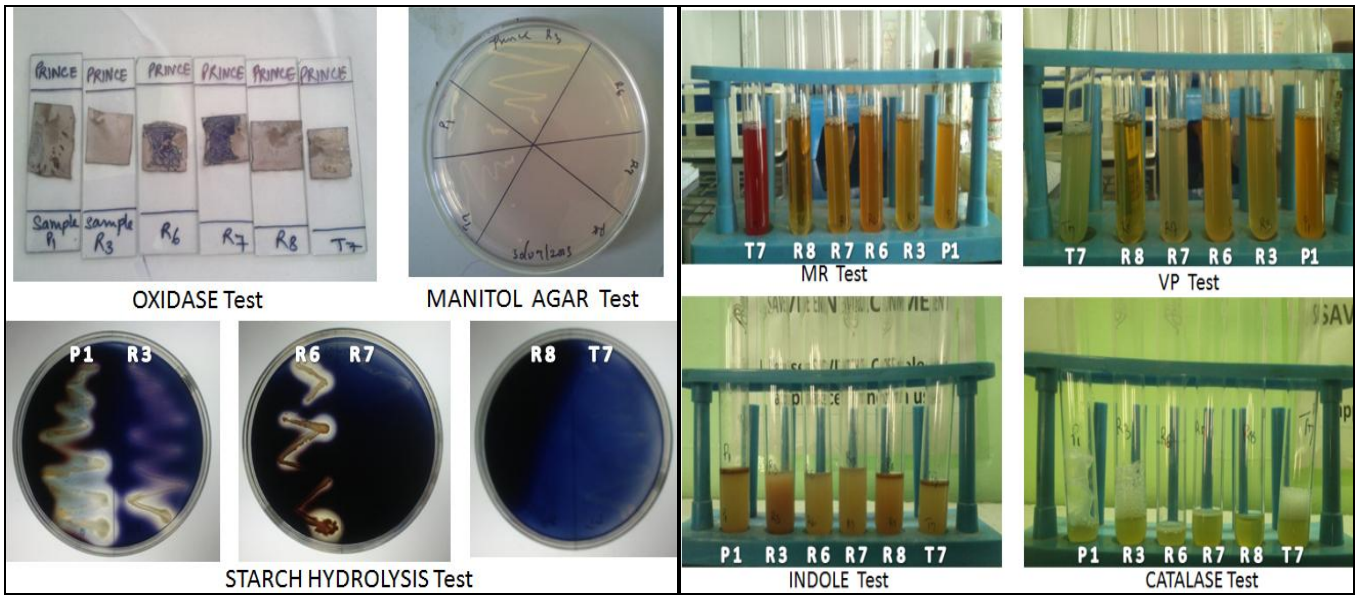


FIG. 7: ZONE OF INHIBITION APPEARED IN P1 AGAINST TEST ORGANISMS 5, 6 AND 7

TEST ORGARNISMS- 1- *S. aureus*, 2- *E. coli*, 3- *P. aeruginosa*, 4- *B. amyloliquefaciens* , 5- *L. bulgaricus*, 6- *L. fermentum*, 7- *M. luteus*

IDENTIFICATION OF ISOLATES:

Colonial Morphology and Characterization of Isolates: The colonies formed after incubation of each isolate were examined for the type of growth, shape, elevation, size, pigmentation and consistency. Microscopic examination was done with a compound microscope. Selected strains P1, R3, R6, R7, R8 and T7 were examined by Gram stain, and identified by standard bacteriological and biochemical methods given by Holt. Taxonomical characterization of selected isolates were done by testing for IMViC test, Urease, Salt concentration, Gelatin liquefaction, Starch hydrolysis, Catalase test, Oxidase test, Carbohydrate Assimilation Test and Mannitol Salt agar test as seen in **Figure 8**. The bacterium which shows maximum bacteriocin activity was further identified.



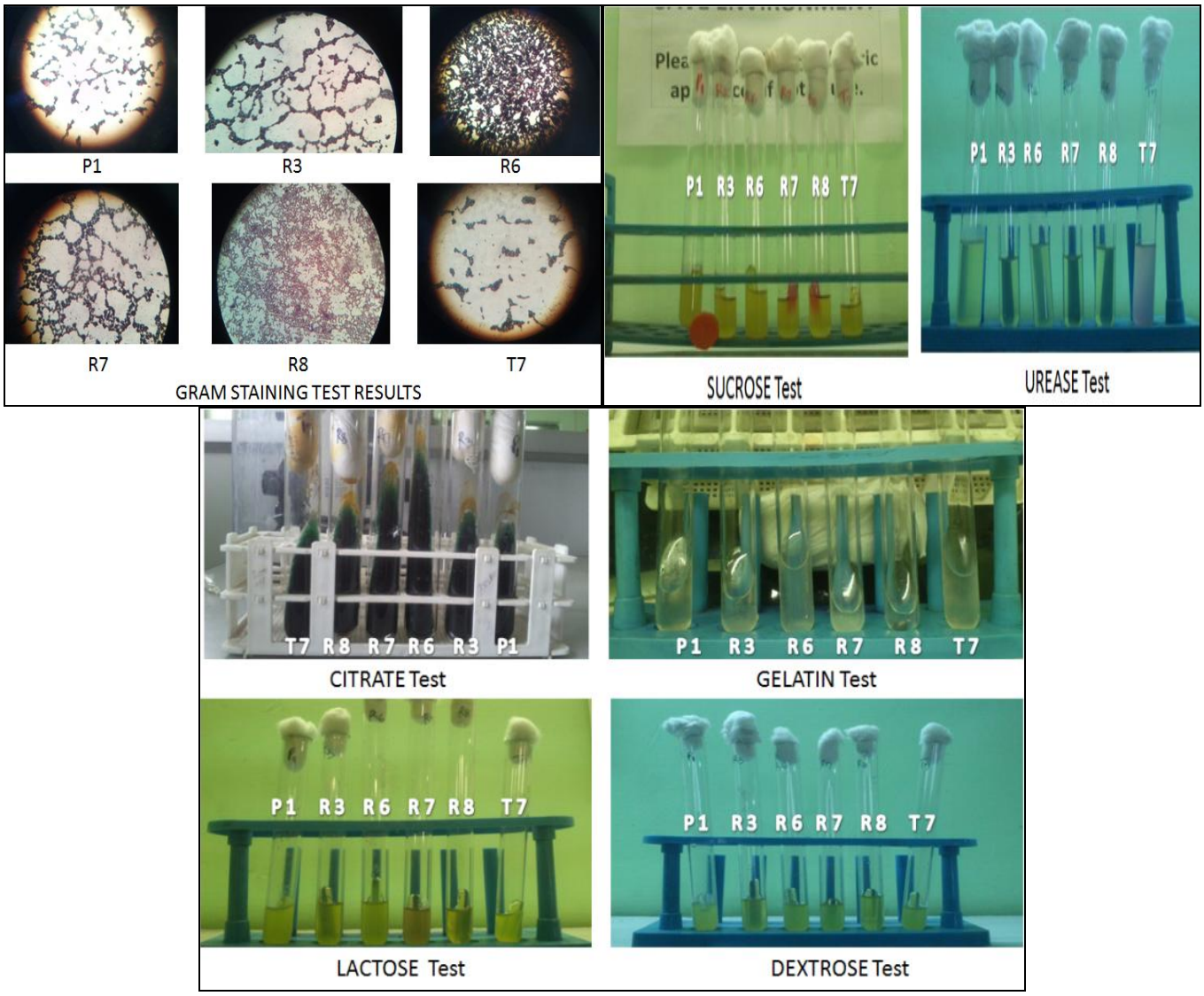


FIG. 8: TAXONOMICAL AND BIOCHEMICAL CHARACTERIZATION OF SELECTED ISOLATES

RESULTS AND DISCUSSION: A total of nineteen colonies were randomly picked from the various samples. They were picked based on distinct colonial characteristics. They were all subjected to bacteriocin assay and the strains showing a relative positive result and the diameters were measured as described in **Table 1**. A total of six isolates were selected and subjected to morphological, physiological and biochemical tests which include Gram's staining, Catalase Test, Oxidase Test, Indole Test, Methyl Red Test, Voges Proskaeur Test, Gelatin hydrolysis, fermentation of sugars, growth at different pH and at 4% and 10% Nacl concentration described in **Table 2**. The bacteriocin isolated from P1 showed maximum preservative effect in raw milk up to 4 days 1 without any change in pH and color as shown in **Figure 9**. After 4 days pH started going down to acidic and color of milk turns to yellow with respect to the control milk sample which became acidic the next day and showed a change in its color.

Hernandez reported that several *Lactococci* produced nisin like activity and showed a broad inhibitory spectrum against the indicator strains tested[9].The growth of *S. aureus*in foods presents a potential public health hazard. A total of 285 samples of meat and meat products were evaluated by Bromberg *et al* for the presence of bacteriocin-producing lactic acid bacteria. The results of the well-diffusion assay showed that 64.1% of the isolated strains only inhibited *S. aureus*, and 11.7% only showed inhibitory activity against *L. innocua*[10].

TABLE 1: ZONES OF BACTERIOCIN PRODUCED BY STRAINS AGAINST INDICATOR ORGANISMS. (MM)

Indicator Organisms	P1	R3	R6	R7	R8	T7
Staphylococcus aureus	+(2)	+(0.5)	+(2)	+(2)	+(1)	+(1)
Escherichia coli	+(2)	+(0.5)	+(2)	+(4)	+(3)	+(3)
Pseudomonas aeruginosa	+(2)	+(0.5)	-	+(3)	+(1)	-
Bacillus amyloliquefaciens	+(2)	-	+(4)	+(4)	+(3)	+(3)
Lactobacillus bulgaricus	+(4)	+(1)	+(2)	+(1)	-	-
Lactobacillus fermentum	+(8)	+(3)	-	+(1)	-	-
Micrococcus luteus	+(4)	-	-	-	-	-

+ Sensitive; - Resistance; 5-9mm Strong Inhibition; 0.5-4mm; Weak Inhibition

TABLE 2: BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERIZATION OF BACTERIA ISOLATES SHOWING BACTERIOCIN ACTIVITY

Isolates	Morphology	Gram's Reaction	Catalase	Oxidase	Citrate	Indole	Methyl Red	V.P	Urease	at pH 4.5	at pH 9.6	4%NaCl	10%NaCl	Homo/Hetero	Dextrose	Lactose	Sucrose	Manitol Agar
P1	rods	+	+	-	-	+	-	-	-	+	+	+	+	HT	+	+	+	-
R3	rods	+	+	-	-	-	-	-	-	-	+	+	+	HM	+	+	+	+
R6	cocci	+	+	+	-	-	-	-	-	+	+	+	+	HT	+	+	+	-
R7	rods	+	+	+	-	-	-	-	-	+	+	+	+	HM	+	-	+	-
R8	rods	-	-	-	-	+	-	-	-	-	+	+	+	HM	+	+	+	-
T7	rods	+	+	-	-	+	+	-	+	-	+	+	+	HM	+	+	+	+

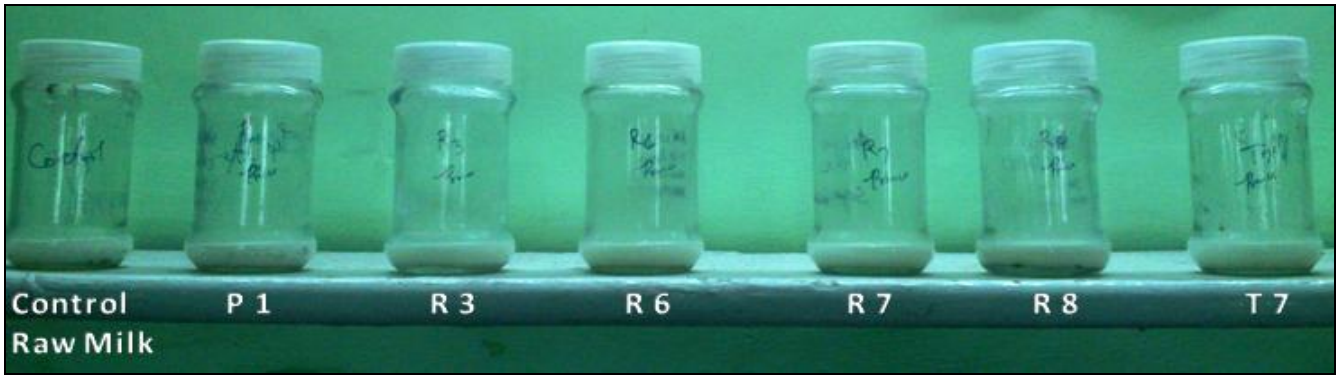


FIG. 9: BACTERIOCIN EFFECT ON RAW MILK PRESERVATION

CONCLUSION: In this study, the isolated microbe showed bacteriocin production and its effect against different microbial species, these microbes have been isolated from different sources such as curd, milk and soil. The selected isolated strain producing bacteriocins have been further characterized. Among all six isolates P1, R3, R6, R7, R8, T7, Bacteriocin activity was found in P1and R7 strains against various test organisms which showed a promising result. Best bacteriocin producing microbe strain P1 was identified as *Paenibacillus lactis* which is an ideal candidate for higher yielding and enhancement of bacteriocin production and use.

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