

## Isolation and Characterization of Bacteriocin Producing Lactic Acid Bacteria from Curd

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**Abstract:** Bacteriocins are proteinaceous antibacterial compounds, which constitute a heterologous subgroup of ribosomally synthesized antimicrobial peptides. In this project a total of 20 colonies were isolated from curd sample taken from the Vignan's Hostel Mess, Vadlamudi, Guntur (Dt), Andhra Pradesh. 6 colonies showed catalase negative and among 6 colonies 3 showed Bacteriocin activity against *E. coli*, *Staphylococcus aureus*, *Acinetobacter sps*, and *Acetobacter aceti*. There is no effect on *Bacillus cereus* and *Streptococcus sps*. The 3 colonies were named as strain 9, strain 10 and strain 13. Among 9, 10 and 13 the highest antimicrobial activity against *E.coli*, *staphylococcus sps* *Acetobacter aceti* and *Acinbetobacter sps* was observed for strain 13. The strain 13 showed strong inhibition in acidic pH compared with basic pH. The maximum activity was observed against *Acinetobacter sps* and *Acetobacter aceti*. Three strains showed antibiotic resistance to Amikacin, Ampicillin and Chloramphenicol. Thus the strains may be used as probiotic products.

**Key words:** Bacteriocins, Anti microbial peptides, Antimicrobial Activity, Probiotics.

### Introduction

Probiotics, because of their role in the maintenance of normal gastrointestinal flora can facilitate human resistance to opportunistic infection. The term "friendly bacteria" is often used as an alternative term for "Probiotics." The word "Probiotic" actually means life. World Health Organization (WHO) defined as the "Live microorganisms that provide a health benefit to the host when ingested in adequate amounts." It was invented in early 20<sup>th</sup> century by Eli Metchnikoff, and he introduced in his study<sup>1</sup>. These beneficial microorganisms are as a component of fermented foods are an integral part of the human diet. The widely used probiotic bacteria are the lactic acid bacteria (LAB) especially the genera of *Lactobacillus* and *Bifidobacterium* species. FDA considered these organisms as "GRAS" (generally regarded as safe) which includes *Lactobacillus sps*, *Bifidobacterium*, *Lactococci* and *yeast*. There are other organisms, such as *Enterococcus*, *Bacillus*, *Streptococci* and other spore-forming bacteria are not generally regarded as safe but have been used as probiotics<sup>2,3</sup>. Starter bacteria used in yogurt cultures include *Lactobacillus bulgaricus* and *Streptococcus thermophilus*; however, it is not clear whether these bacteria are capable of colonization of the human GI tract. The useful compounds produced by Lactic acid bacteria are exopolysaccharides<sup>4,5</sup>, bacteriocins<sup>6</sup>, free aminoacids, short chain fatty acids, vitamins<sup>7</sup>, digestive enzymes and oligosaccharides. Bacteriocins are proteinaceous antibacterial compounds, which are ribosomally synthesized antimicrobial peptides<sup>5</sup>. These bacteriocins have novel applications other than food preservation. Various microorganisms were adapted to antibiotics by several mechanisms and they become as multidrug resistant bacteria. The mode of action of bacteriocins is differing from conventional antibiotics so they are considered as novel source for the control of

these microbial pathogens. Bacteriocins are produced by both gram positive and gram negative bacteria<sup>7</sup>. Bacteriocin from Lactic acid bacteria exhibit a broad spectrum of activity includes protozoa, fungi, and yeast<sup>8</sup>.

Some of the bacteriocins are cytotoxic, with activity against sperm and tumor cells. This feature makes them attractive for formulation in feminine health care and contraceptive products<sup>8, 9</sup>. The bacteriocins produced by lactic acid bacteria are widely used in medical and personal care applications. In addition to all these applications the antioxidative activity of lactic acid bacteria was also reported<sup>10</sup>. Extensive use of antibiotics in human health for various diseases results in the emergence of antibiotic resistance bacteria as well as multi drug resistant pathogens. The products produced by lactic acid bacteria exhibiting antimicrobial activity especially bacteriocins, hydrogen peroxide, enzymes and lactic acid helps in prevention and therapeutic purposes in controlling the pathogens<sup>10</sup>.

In this present study bacteriocin producing LABs were isolated from curd sample and their morphological and biochemical tests were carried out. The bacteriocin activity was assayed by agar well diffusion method. The bacteriocins activity was checked for different temperatures and pH. Antibiotic resistance profile was carried out for knowing the potential as probiotic.

## Materials and Methods

Antibiotic discs were purchased from Hi-media Laboratories, Mumbai, India. Chemicals were purchased from S. D. Fine-Chem. Ltd., Mumbai, India. All these chemicals were of analytical grade.

### Sample collection and isolation of pure cultures:

Curd sample was collected in sterile bottle from the Vignan's Hostel Mess, Vadlamudi, Guntur (Dt), Andhra Pradesh. 1 gm was dispensed in 10ml of sterile distilled water. This is mixed vigorously and 1ml from this tube was taken and added to another tube containing 9 ml of water to get a dilution of  $10^{-1}$ . This serial dilution is repeated up to  $10^{-9}$  dilutions. For the isolation of organisms 0.1ml of each dilution was plated on MRS medium by spread plate method and the plates were incubated at 37°C for 24-48hrs to allow microbial growth. Pure cultures were then developed for the selected colonies using MRS medium by streak plate method and incubated at 37°C for 24hrs and then stored at 4°C.

### Indicator strains:

The indicator strains used to check the Bacteriocin activity were *Escherichia coli*, *Staphylococcus aureus*, *Acinetobacter sps*, *Acetobacter aceti*, *Bacillus cereus* and *Streptococcus sps*. Except *Acetobacter aceti* all the cultures were preserved in Nutrient agar slants at 4°C and the *Acetobacter aceti* was preserved in specific medium at 4°C.

## Screening of Lactic acid bacteria for Bacteriocin Production

### Agar Well Diffusion Method:

Antimicrobial activity of the bacterial isolates against all the indicator strains was determined by well diffusion method under aerobic conditions. The inhibitory activity against *Acetobacter aceti* was tested on its specific media where as *E. coli*, *Staphylococcus aureus*, *Acinetobacter*, *Streptococcus* and *Bacillus cereus* were tested on nutrient agar media. Agar plates were inoculated with 100 µL of each target microorganism after growing them in a broth for overnight by spreading method. The plates were allowed to dry and a sterile cork borer of diameter 7.0 mm was used to cut uniform wells in the agar plates. Lactic acid bacteria were grown in MRS broth at 37°C for 24h. After incubation, cells were removed by centrifugation at 10,000 rpm for 10 min. The supernatant was adjusted to P<sup>H</sup> 6.5 to 7.0 with 1N NaOH and filtered through 0.22 µm membranes. Each well was filled with 100 µl of filter-sterilized supernatant obtained from culture grown in MRS medium. Incubate the plates at 37°C for 24h. After incubation the antimicrobial activity was determined by measuring the diameter of inhibition zone around the wells. The bacterial isolate showing the widest zone of inhibition against the target microorganism was selected for further studies<sup>7</sup>.

### Morphological and Biochemical characterization of isolates:

Bacteriocin producing isolates were identified and characterized by morphological and biochemical characterization according to the Bergey's Manual of Determinative Bacteriology (Krieg and Holt 1984).

Morphological features were identified by growing the cultures on MRS agar media and gram staining was performed for each isolate. Different Biochemical tests were carried out includes IMVIC tests, catalase test, starch hydrolysis and carbohydrate fermentation of various sugars <sup>11</sup>.

### **Growth Curve for Bacteriocin producing isolates:**

MRS medium was prepared for the selected strains and sterilized by autoclaving at 121°C for 15min. After cooling a 1% inoculum was added to the broth and incubated for 48hrs. For every 4hrs interval, the optical density of each broth was measured using spectrophotometer at 660nm.

### **Characterization of Crude Bacteriocin**

#### **Effect of Temperature:**

MRS medium was prepared for three strains and sterilized by autoclaving at 121°C for 15min. After cooling a 0.1% inoculum was added to the broth and incubated for 24hrs at 37°C. After incubation the culture was centrifuged at 10000 rpm, 4°C for 10 min. The cell free supernatant was adjusted to pH 6.5 to 7.0 with 1N NaOH. This supernatant was exposed to different temperatures such as 25°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 90°C, 100°C and 121°C for 30min and then assayed for bacteriocin activity.

#### **Effect of pH:**

MRS medium was prepared for the three strains and sterilized by autoclaving at 121°C for 15min. After cooling a 0.1% inoculum was added to the broth and incubated for 24hrs at 37°C. After incubation the culture was centrifuged at 10000 rpm, 4°C for 10 min. The cell free supernatant was adjusted to pH 2, 3, 4, 5, 6, 7, 8, 9 with Hydrochloric Acid (1N HCl) and Sodium Hydroxide (1N NaOH), incubated for 1hr at room temperature and then assayed for bacteriocin activity using agar well diffusion method.

#### **Antibiotic resistance:**

Antibiotic resistance of isolated lactic acid bacteria strains were assessed using antibiotic discs (Hi media Laboratories Pvt. Ltd. Mumbai, India) on MRS agar plates. The antibiotic discs Amikacin, Ampicillin and Chloramphenicol were placed on the surface of agar plate and pressed. Allow the plates to diffuse the antibiotic into the medium for 1hr. Incubate the plates for overnight and observe the zone of inhibition<sup>12</sup>.

## **Results and Discussion**

### **Isolation of Bacteriocin Producing Bacteria:**

In the present study, Lactic acid bacteria (LAB) were isolated from the curd by serial dilution method. 20 colonies with different morphology were isolated on MRS agar medium. The colonies are selected from 10<sup>-3</sup> and 10<sup>-4</sup> dilutions. These colonies were maintained as pure cultures by streak plate method in MRS agar slants at 4°C.

### **Screening of Bacteriocin Producing Bacteria:**

The bacteriocin activity was checked by using agar well diffusion method. The indicator strains used to check the bacteriocin activity were *Escherichia coli*, *Staphylococcus aureus*, *Acinetobacter sps*, *Acetobacter aceti*, *Bacillus cereus* and *Streptococcus sps*. Among 20 colonies the strains showing catalase negative were selected for bacteriocin activity. 6 colonies showed catalase negative and were named as strains – 9, 10, 13, 14, 18 and 20. Among 6 colonies 3 showed bacteriocin activity against *Escherichia coli*, *Staphylococcus aureus*, *Acinetobacter sps*, and *Acetobacter aceti*. There is no effect on *Bacillus cereus* and *Streptococcus sps*. The 3 colonies were named as strain 9, strain 10 and strain 13. The strains-14, 18 and 20 did not show any inhibitory activity.

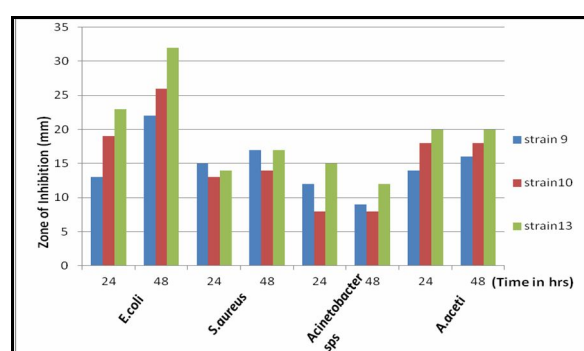
After 24hrs of incubation strains- 9, 10, 13 showed strong inhibition against *E.coli*, *Staphylococcus aureus* and *Acetobacter aceti* where as weak inhibition against only on *Acinetobacter sps*. Among these three, strain 13 showed maximum inhibitory activity. Strain 10 has less activity on *Acinetobacter sps*.

After 48hrs of incubation strain 9, 10 and 13 showed maximum inhibitory activity against *E. coli* and *S. aureus*. *Acinetobacter sps* showed very less activity compared with 24hrs. In case of *Acetobacter aceti* the

activity was increased for only strain 9 and there is no change in the case of strain 10 and 13. The inhibition zones (mm) were depicted in Table 1. The highest antimicrobial activity in terms of inhibition zone was observed for bacteriocin produced from strain 13 has 32mm and was shown in figure 1. Earlier the bacteriocin activity was observed for 23mm for the isolates from fermented vegetables<sup>13</sup>.

**Table 1: Bacteriocin activity (Zone of inhibition) against indicator strains for 24hrs and 48hrs.**

Bacteriocin activity (Zone of inhibition in mm)								
Name of the organism	<i>E. coli</i>		<i>S. aureus</i>		<i>Acinetobacter sps</i>		<i>Acetobacter aceti</i>	
	24hrs	48hrs	24hrs	48hrs	24hrs	48hrs	24hrs	48hrs
Strain 9	13	22	15	17	12	09	14	16
Strain 10	19	26	13	14	08	08	18	18
Strain 13	23	32	14	17	15	12	20	20



**Figure 1: Bacteriocin activity of strains 9, 10 and 13 against *Escherichia coli*, *S. aureus*, *Acinetobacter sps* and *Acetobacter aceti*.**

### Morphological and Biochemical characterization of isolates

Morphological features were observed for the isolates grown on MRS agar medium. Strain 10 and 13 were bacilli in shape where as 9 was cocci in shape. All the three strains were Gram positive and white transparent in color. These are represented in Table 2. Biochemical tests were represented in the figures 2 & 3.

**Table 2: Morphological and Biochemical characteristics of strain 9, 10 and 13**

	Strain 9	Strain 10	Strain 13
<b>Morphological features</b>			
Gram staining	Gram +ve	Gram +ve	Gram +ve
Shape	Cocci	Bacillus	Bacillus
color	White transparent	White transparent	White transparent
<b>Biochemical tests</b>			
Indole Production	Negative	Negative	Negative
Methyl Red	Positive	Positive	Positive
Voges-Proskauer	Negative	Negative	Negative
Citrate Utilisation	Negative	Negative	Negative
Catalase	Negative	Negative	Negative
Starch Hydrolysis	Negative	Negative	Negative
<b>Carbohydrate Fermentation</b>			
Dextrose	Negative	Positive, Acid producer	Positive, Acid producer
Glucose	Positive, Acid producer	Positive, Acid producer	Positive, Acid producer
Fructose	Positive, Acid producer	Negative	Positive, Acid producer
Mannitol	Negative	Negative	Positive, Acid producer

Sucrose	Positive, Acid producer	Positive, Acid producer	Positive, Acid producer
Lactose	Negative	Positive, Acid producer	Positive, Acid producer

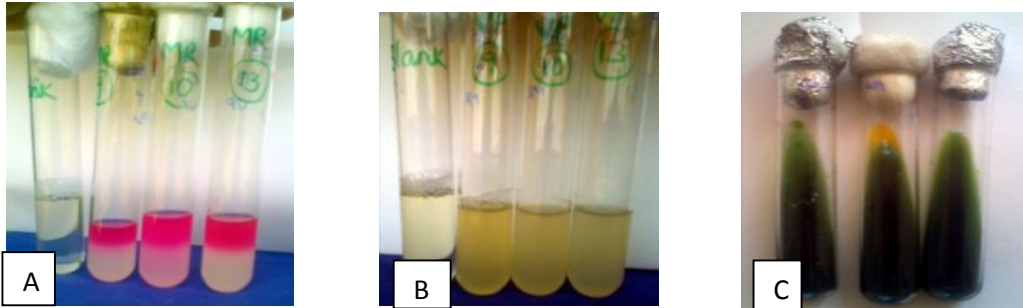


Figure 2: Images of Biochemical tests A) Methyl red test: Strains 9, 10 and 13 were positive to MR test B) VP test: Strains 9, 10 and 13 were negative for VP test C) Citrate utilization test: Strains 9, 10 and 13 were negative for Citrate utilization test.

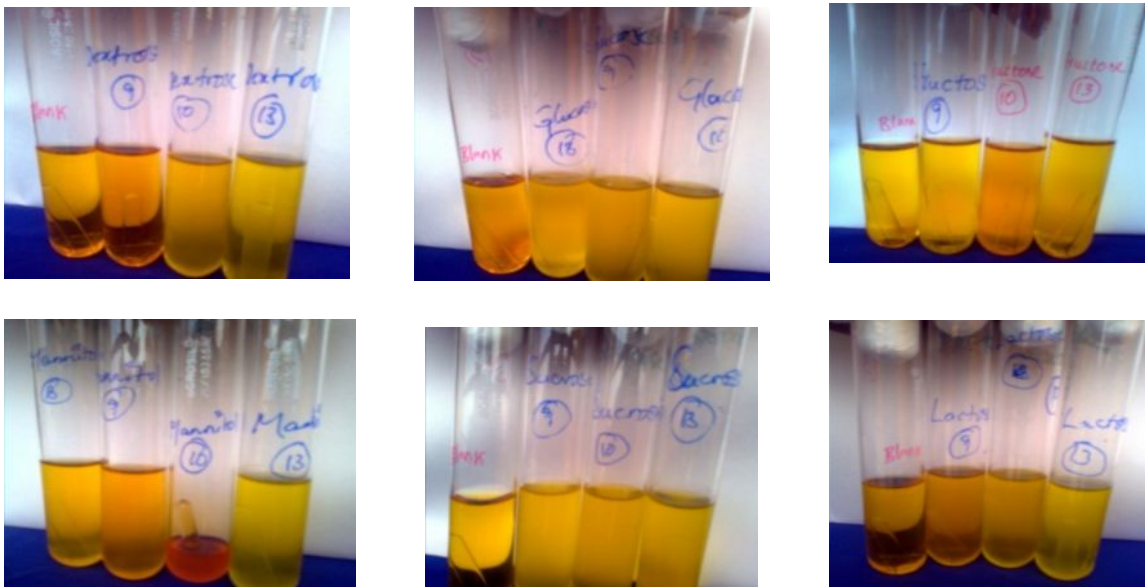


Figure 3: Carbohydrate fermentation tests of strain 9, 10 and 13: A) Dextrose B) Glucose C) Fructose D) Mannitol E) Sucrose F) Lactose

In carbohydrate fermentation test strain 9 showed negative for dextrose, mannitol and lactose fermentation where as positive, acid producer for glucose, fructose and sucrose. Strain 10 showed negative for fructose and mannitol fermentation and positive, acid producer for dextrose, glucose, sucrose and lactose. Strain 13 showed positive acid producer for all the carbohydrate fermentations.

**Growth Curve**

Bacteriocin producing isolates were grown in MRS medium for 48hrs at 37°C. Optical readings were taken at 600nm for every 4hrs time interval. Growth curve was plotted by taking time on X-axis and O.D on Y-axis.

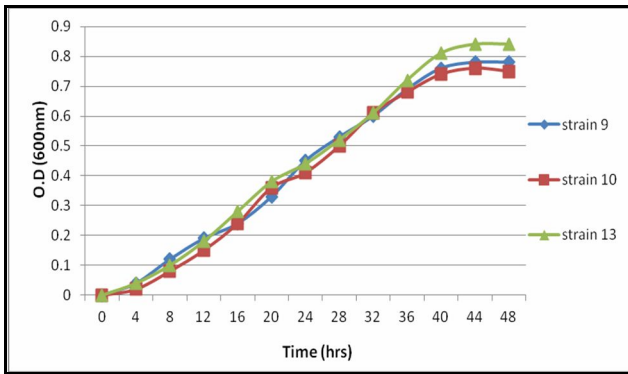


Figure 5: Growth curve for strains 9, 10, 13

Effect of temperature

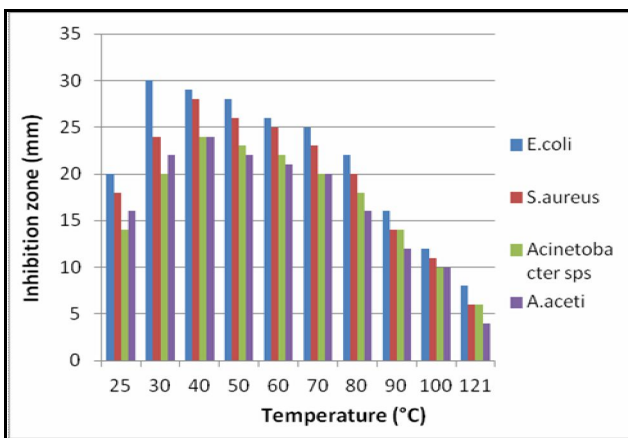


Figure 6 (a): Strain 9 Bacteriocin activity at different temperatures

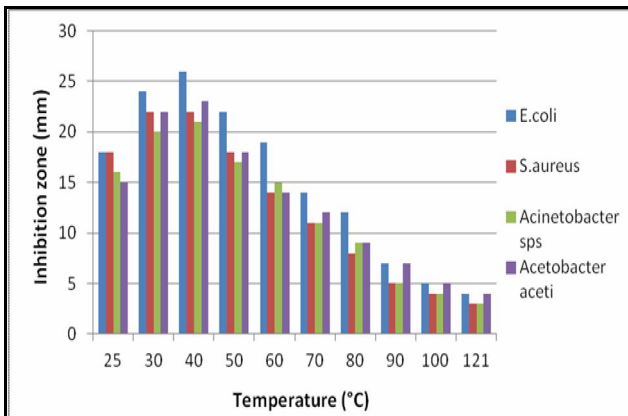


Figure 6 (b): Strain 10 Bacteriocin activity at different temperatures

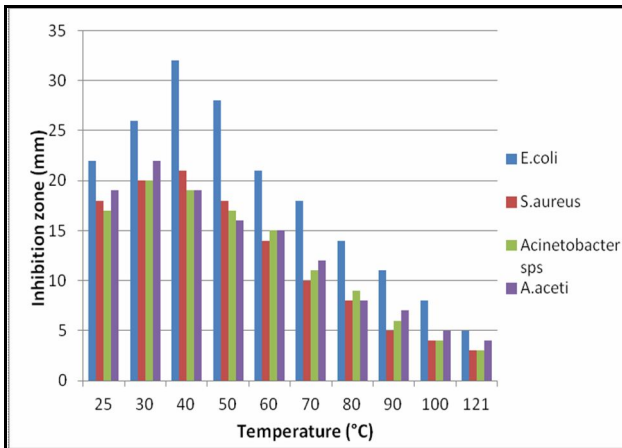


Figure 6 (c): Strain 13 Bacteriocin activity at different temperatures

In figures 6 (a), (b), (c), represents the effect of temperature on bacteriocin activity in terms of inhibition zones for strains 9, 10 and 13. The maximum activity of bacteriocin was observed 30-50 range of temperature for the three strains. With the increase of temperature the bacteriocin activity was decreased and it cannot withstand at high temperatures such as 100°C and 121°C. There is a partial loss of activity was observed with the increase of temperature. Strains 9 and 13 were more active at temperatures of 30-50 range and strain 10 showed less activity compared with strains 10 and 13.

Effect of pH

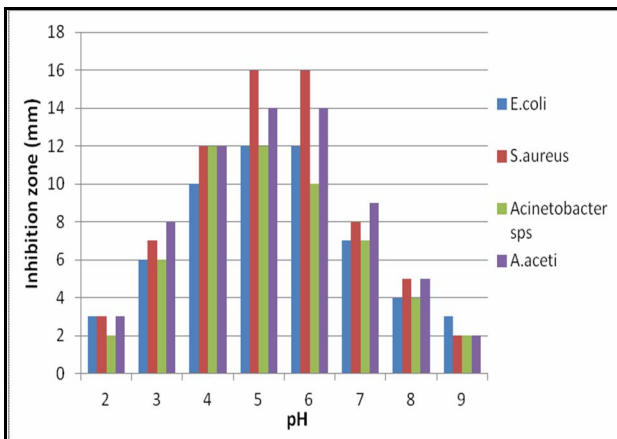


Figure 7 (a): Strain 9 Bacteriocin activity at different pH

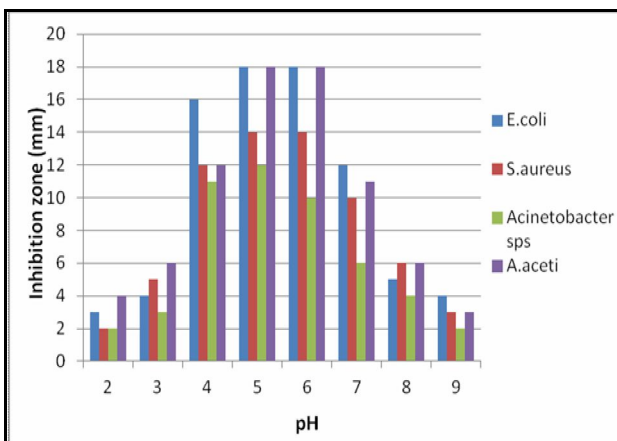
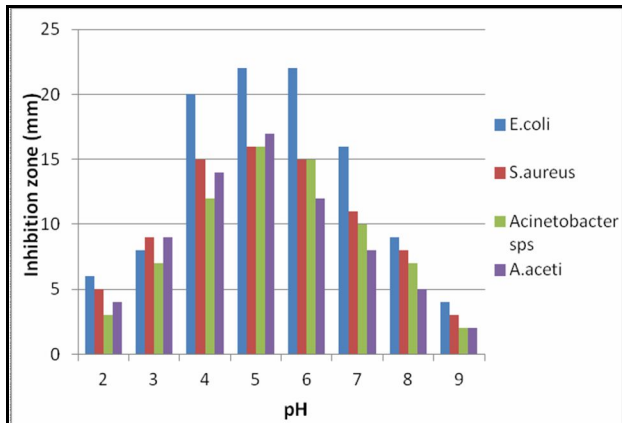


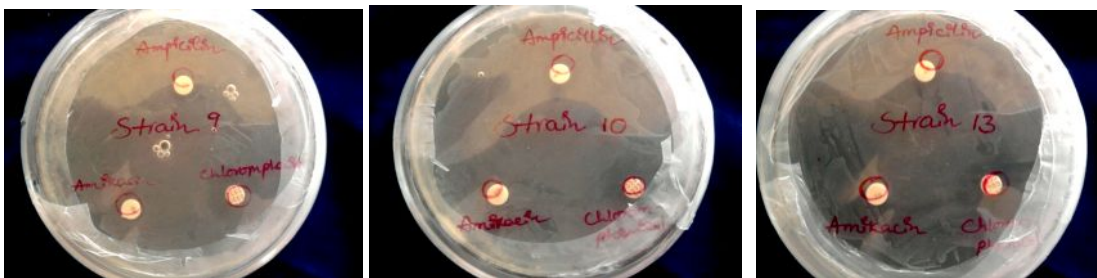
Figure 7 (b): Strain 10 Bacteriocin activity at different pH



**Figure 7 (c): Strain 13 Bacteriocin activity at different pH**

Among the three strains bacteriocin produced by strain 13 showed more activity compared with 9 and 10. The effect of pH on bacteriocin activity was measured in terms of inhibition zones. In figures 7 (a), (b), (c) represents the effect of pH on bacteriocin activity for three strains. For all the three strains maximum activity was observed from slightly acidic to neutral pH. With the increase of pH the antimicrobial activity was decreased. Based on these results slightly acidic to neutral pH was suitable for bacteriocin activity.

### Antibiotic Resistance



**Figure 8: Antibiotic resistance of strains 9, 10 and 13 against Ampicillin, Amikacin and Chloramphenicol**

All the three strains are positive to the test i.e. they are resistant to those antibiotics. Thus, the resistance indicates that if isolated probiotics induced in patients treated with antibiotic therapy may be helpful in faster recovery of the patients due to rapid establishment of desirable microbial flora.

### Conclusions

The main aim of this study was to isolate the bacteriocin producing lactic acid bacteria and checking their antagonistic activity and potential as good probiotic characteristics. The isolated LABs producing bacteriocins has high antagonistic activity against *E.coli* and *Staphylococcus aureus* pathogens. These pathogens causing very severe infections in the intestine and urinary tracts and become as multidrug resistant pathogens. The bacteriocins has showed strong inhibitory activity against these pathogens as well as resistance to antibiotics helps in the preventive and therapeutic purposes in the clinical conditions.

### Acknowledgements

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