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# CHARACTERIZATION OF BACTERIOCIN FROM PROBIOTIC *LACTOBACILLUS PLANTARUM*

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## ABSTRACT

In our study, the sample (cheese) was selected for isolation and identification of *Lactobacillus* species as local probiotic isolate. The strain was subjected to microscopic and macroscopic investigations for probiotic selection. The world health organization criteria (WHO) were applied to all *Lactobacillus* species against *E. coli, P. areoginosa, S. aerus, Chromobacterium, Serratia and A. flavus*, antibiotic sensitivity test, acid and bile tolerance test, heamolytic activity. Antimicrobial compound called bacteriocin was partially purified. The *Lactobacillus* species was identified by 16S rRNA gene sequencing and specific catalase gene was also amplified. Data showed that *Lactobacillus plantarum* has a high inhibitory activity, tolerant to bile and acid, highly resistant to many antibiotics. Antimicrobial peptide was partially purified, characterized and bacteriocin produced by *L. plantarum* remained constant activity after heating at 121$^{o}$C for 10 min. *L. plantarum* may be an alternative and promising way for eradicating many diseases. *L. plantarum* produces antimicrobial metabolites can give reasonable assurance of the control of pathogenic microorganisms. Increase the number of food-poisoning patients, the inhibition of the bacterial growth or production of enterotoxin such as verotoxins by administrating Lactobacilli bacteriocins would be of great importance. *Lactobacillus* fulfills the basic criteria required for probiotic strains which survive in *in-vitro* condition. The experimental strain exhibited strong antibacterial and antifungal activities against the pathogens. *L. plantarum* has high probiotic potential for eradicating many diseases, mainly the suppression of *A. flavus* which produce Aflatoxin. Pharmaceutical and nutritional industries are exploring more natural treatments for health conscious consumers as natural treatments have been effective.

**INTRODUCTION:** Probiotics (Greek word “for life”) fall within the category of functional foods. They are food products containing live microorganisms that exert beneficial health effects in host by improving microbial balance in the intestine. Probiotic deficiency leads to constipation, diarrhea, flatulence, liver damage and cancer. Probiotics are live microbial food ingredients which has beneficial effect to human health. They are present to exert such effects by changing the composition of the gut microbiota. Several probiotics preparation seems to have promise in prevention or treatment of various disease conditions.

At least 500 different microbial species exist in the GIT although on a quantitative basis about 20 genera probably predominant. These include bacteriods, *Lactobacillus, Clostridium, Fusobacterium, Eubacterium, Peptococcus, Peptostreptococcus, Esrichia* and *Veillonella*. The bacterial strains with beneficial properties include mainly *Lactobacilli* and *Bifidobacteria* [1].

Lactic acid bacteria (LAB) comprise a wide range of genera and include considerable nutrients of species. Their common traits are: gram positive, usually catalase negative, grown under micro aerophillic to strictly anaerobic conditions and lactic acid production. These bacteria are the major component of the trasters used in fermentation, especially for dairy of the gastrointestinal microflora. *L. plantarum* 299v leads to significant reductions in fibrinogen and LDL-cholesterol concentrations in smokers. The reduced systolic blood pressure were observed in the *L. plantarum* group is of potential clinical significance [2] There is a wide number of bacteriocins produced by different LAB, and they can be classified according to their biochemical and genetic characteristics.

The *class IIb* is formed by a complex of two distinct peptides. These peptides have little or no activity and it appears to be no sequence similarities between complementary peptides. In this group are lactococcin G and plantaricins EF e JK [3].

**METHEDOLOGY:**

**Sample collection and maintenance:** Bacterial strain was obtained from diary product-cheese and fungal strain was obtained from MTCC culture lab, Chandigar, India. For experimental work take 1g of sample was weighed aseptically, were serially diluted.0.1ml of the serially [diluted] samples were spread plated over the surface of the MRS agar plates. The plates were incubated at 37˚C for 48-60 Hrs. The colonies were observed. Stock cultures were prepared by growing the strains for 16-24 h and inoculating 0.5ml into 0.5ml of sterile 80% glycerol.

**Biochemical characterization**

1. **Gram's staining:** Bacterial smear was prepared by heat fixation. Then the slide was flooded with crystal violet staining reagent for 1 minute and wash the slide. Then Gram's iodine was added for 1 minute and again washed for 2 seconds. Then smear was blotted dry with absorbent paper. The smear was immersed in 95% ethanol for 30 seconds with gentle agitation and washes the smear. The smear was immersed for 2 min with counter stain and washes the smear until the colour was removed. The smear was dried with the absorbent paper and examined under the microscope.

2. **Motility determination- Hanging Drop experiment:** A cover glass and a cavity slide were cleaned with alcohol. A thin film of Vaseline was placed around the rim of the cover glass. A loop of log phase culture was placed in the center of cover glass. The cavity slide was hold inverted, cavity down the cover glass then pressed it so that the Vaseline adhered to the slide. The slide was turned carefully down to make the drop hanging in the cavity. Then the edge of the drop was observed under a high power oil immersion microscope.

3. **Methyl Red Test:** Culture were inoculated in two sets of test tubes containing MR-VP broth and incubated for 2 days at 30$^{o}$C.to one set of tube approximately 5-10 drops of methyl red solution has been added and allowed to run slowly down side the tube to form a layer on the surface of the culture.

4. **Catalase Test:** A loopful of culture grown on nutrient agar plate for 24-48 hrs was taken. Then the loop was placed in 1% $H_2O_2$ on a glass slide, and then the slide was observed for the production of air bubbles with effervescence.

5. **Arginine Hydrolase Test:** A loopful of culture grown on arginine agar as stab culture in a screw cap vial, incubated for 4-7 days.

6. **Growth at different Temperature:** Overnight isolated cultures were inoculated at 10%(v/v) in MRS broth and incubated at 15$^{o}$C, 37$^{o}$C,and 45$^{o}$ C for 24 h. Total populations were determined by the pour plate method, incubating the plates at 37$^{o}$C for 48 h.

7. **Growth at different NaCl concentration:** Overnight isolated cultures were inoculated at 10

%( v/v) in MRS broth with 5% NaCl, 7% NaCl, 10%NaCland incubated at 37°C. Total populations were determined by the pour plate method, incubating the plates at 37°C for 48 h.

**Characteristic of properties of potentially Probiotic Bacteria:** The experiments were done for characterizing the capability to metabolize saccharides, resistance to antibiotics, antagonistic effect of bacteria against selected microorganisms, resistance to bile salts and survivability in the environment with different pH of all 3 strains of potentially probiotic bacteria.

**Carbohydrate Fermentation Profile:** Carbohydrate fermentation broth were prepared, dispersed in tubes and sterilized with Durham's tubes. After sterilization 1ml of 10% filter sterilized respective sugar solution to be fermented were added. The sample were inoculated and incubated at 37°C for 24Hrs.

**Antibiotic Susceptibility Test:** 50µl of the pellet of an overnight culture was diluted in MRS broth Muller-Hinton agar plates were flooded with this suspension and air dried for 15 min, and the disks impregnated with antibiotics were positioned on the plates. After 36 h of incubation at 37°C in air containing 5% $CO_2$, the diameters of the bacteria-free zones were measured.

**Acidity Resistance Test:** The pH of MRS medium was adjusted to following values: 3; 4; 5; 6; 7; 8. The different pH were obtained by using 1 M HCL and 0.5 M NaOH, the three samples are inoculated in different pH containing MRS medium. The number of live bacteria (CFU/ml) was measured at definite time intervals (24Hrs, 48Hrs, 72 Hrs, 96Hrs). The test was replicate twice.

**Bile Tolerant Test [4]:** Acetyl choline was used as the equivalent of bile in this experiment. The following concentration of acetyl choline in the medium was used: 0, 0.1, 0.2, 0.3, and 0.4% (w/v) and they corresponded to tenfold higher concentration of bile. The added bacterial inoculum was of 10% (v/v) of the medium and the culture were incubated anaerobically at 37°C for 8 h. bacterial growth was measured spectrophotometrically at 620 nm at 2Hrs intervals during the 8Hrs incubation period.

Appropriate controls (MRS and Medium without culture) were used as a reference blank. To quantify inhibition of culture by bile, a coefficient of inhibition was calculated, according to the formula:

Co. of inhibition = (A620 nm control - A620 nm bile): A620 nm control

Where: A620 nm control-optical density of the culture broth without bile.A620 nm bile-optical density of the broth containing bile measured at same time.

**Haemolytic activity:** Blood haemolysis was evaluated on Nutrient agar plates supplemented with 5% sheep blood which were incubated at 37°C for 24 h [5].

**Species identification by Selective Plating method:** MRS broth enriched with 0.2% tween 80 and supplemented with 1% fructose 0.8% casein acid hydrolysate 0.05% cystenine and 1.5% agar (MRS-fructose) which does not contain either glucose or meat extract. Samples were streaked on the MRS-fructose plates and plates were incubated in anaerobic jars at 45°C for 72 Hrs.

MRS broth enriched with 0.2% tween 80 and supplemented with 1% maltose 0.05% cystenine and 1.5% agar (MRS-maltose).samples were streaked on the MRS-maltose plates and the plates were incubated in anaerobic jars at 37°C for 72Hrs

**Antibacterial activity:**

1. **Preparation of Cell-Free Filtrate:** Samples were inoculated 1% by volume into MRS broth, incubated for 16 Hrs at 37°C.After incubation the 10ml of culture was centrifuged at 15,000rpm for 5minutes. The supernatant were separated and filter sterilized by membrane filter (0.22µm pore size), neutralized the pH of the supernatant to 7.0

2. **Disc Diffusion Method:** Sterile paper discs were prepared and placed on agar plates with indicator strains. 20µl of filtered supernatant was applied and incubated for 24Hrs at 37°C.

**Antifungal activity:**

1. **Disc Diffusion Method:** Sterile paper discs were prepared and placed on agar plates with indicator

strains. 20µl of filtered supernatant was applied and incubated for 24Hrs at 37$^{o}$C.

2. **Suppression of *A. flavus* growth by lab:** 10%V/V (20ML) of LAB inoculum was inoculated into 500l Erlenmeyer containing 180ml LTC medium with or without *A. flavus* incubated at room temperature over 15 days**.** O. D value was obtained at 560 nm.

**Determination of bacteriocin activity [6]:** The inhibitory activity of LAB was screened by agar well diffusion assay (Schillinger and Lucke, 1989). Pre-poured MRS agar plates were overlaid with 7ml MRS soft agar containing 0.2ml of indicator culture

**Partial purification of bacteriocin [7]:**

1. **Ammonium sulphate precipitation:** CFS was treated with solid ammonium sulphate to 0, 30, 35, 40, 45, 50, 55 and 60% saturation. The mixture were stirred for 2h at 4$^{o}$C and later centrifuged at 14,000 g for 1h at 4$^{o}$C. The pellet was resuspended in 25 ml of 0.05 M potassium phosphate buffer pH7.0. Dialysis was carried out against the same buffer for 18 h in spectrapor dialysis tubing. Assay of the bacteriocin activity was carried out and titer was determined.

2. **Trichloroacetic acid (tc) precipitation:** Five percent (5%) equivalent of TC was added to 25 ml of CE1 to precipitate target fraction. The mixture was centrifuged at 12,000g for 10 min, after which the supernatant was decanted. The resulting pellet was dissolved in potassium phosphate buffer, obtaining CE2.

**Characterization of bacteriocin [8]**: The purified bacteriocin samples (Fraction 2) were characterized with respect to thermal and pH stability, susceptibility to denaturation by enzymes, stability during storage, extraction with organic solvent, treatment with dissociating agents and and UV rays induction.

1. **Heat resistance:** Purified bacteriocin (400µl) was exposed to various heat treatments: 40, 60, 80, 100 and 121$^{o}$C. Aliquot volumes of each Fraction were then removed after 0, 30, 60 or 90 min and assayed for bacteriocin.

2. **pH sensitivity:** Purified bacteriocin (400 µl) were adjusted to pH 2, 4, 6, 8, 10, and 12 with hydrochloric acid (HCl) and sodium hydroxide (NaOH), incubated for 4 h at room temperature and similarly assayed.

3. **Enzyme treatments:** Purified bacteriocin was assessed for its sensitivity to various enzymes. Enzymes and their respective buffers were lysozyme (Serva, 20600 U/mg), in 1N NaOH (pH 6.5); proteinase K (11.5 U/mg) in 1N NaOH (pH 6.5). Samples of bacteriocin (500µl) were incubated with 500µg of each enzyme per ml for 60 min at 37$^{o}$C and determined for bacteriocin activity.

**Stability of bacteriocin during Storage:** Purified bacteriocin was stored at –20, 4 and 37$^{o}$C. At different time intervals, samples were taken from the stored material to determine bacteriocin activity.

**Extraction of bacteriocin with organic solvents:** Various organic solvents including iso–amylalcohol, chloroform, n–propanol, hexane, Di ethyl ether, petroleum ether were added to purified bacteriocin in 1:1 ratio. After thorough mixing, phase separation was achieved by centrifugation (10 min at 5000 rpm). When propanol was used for the extraction, 50 g/l NaCl was added to the mixture in order to obtain phase separation. The organic phase and the aqueous phase were collected and solvent removed by evaporation at 45$^{o}$C. The residue from the organic phase was resuspended in an amount of saline (8.5 g/l NaCl) equal to the starting volume of the original supernatant fluid. Bacteriocin activity of both preparations was then determined.

**Effect of UV-light on bacteriocin activity:** A 10 ml aliquot of purified bacteriocin was placed in a sterile petri dish and exposed to short – wave uv light from a 15 – W General Electric germicidal bulb at a distance of 30 cm. Times of exposure ranged from 0 to 5 min. After each time interval, bacteriocin activity was analysed by the well diffusion method.

**Effect of surfactant on bacteriocin activity:** This was carried out by incorporating non-ionic (triton X100, tween 20, tween 80,), anionic (sodium dodecyl sulphate) surfactants were added to purified bacteriocin at a concentration of 0.1 ml or 0.01 g of surfactant ml$^{-1}$ of bacteriocin solutions. These preparations were incubated at 30$^{o}$C for 60 min and

assayed for bacteriocin activity against indicator organisms by using titre evaluation.

**FT-IR:** The purified samples were first dried in an oven at 60$^{o}$C for 4 hours. After removing the moisture content, the samples grown into a fine powder. The IR spectrum of the purified bacteriocin was recorded with a Perkin-Elmer model 297 IR Spectrophotometer. The sample was scanned between 600 and 4000 wave number (cm$^{-1}$) at a speed of 1micron/min, and with a programmed slit opening 2X and air as reference. Infrared spectral analysis of biological material was utilized to investigate chemical constituents. These are recognized even when the amount is material available is very small.

**Molecular size of bacteriocin-SDS page [9]:** Glass plates were assembled and 20ml of 15% resolving gel was prepared and poured immediately to the notch plate. It was overlaid with butanol, after polymerization was completed overlay was poured off and washed the top layer with deionized water. Then 8ml of stack gel was overlaid. Approximate volume of 1×SDS gel loading buffer and sample was taken. Heated it at 100°C for 3 min. Assembly was fixed in electrophoresis apparatus then 15 µL of sample and marker (2,500-40,000 KDa) was loaded respectively in the well, run the gel and stain with Coomassie brilliant blue.

**Isolation of genomic DNA:** The following steps were involved in the genomic DNA isolation.

1.5 ml of culture was taken in a micro centrifuge tube and pelleted out (centrifuged at 10,000 rpm for 5 minutes). The pellet was resuspended in 500 microlitre of TE buffer. 300µl of lysozyme was added and was incubated at 37$^{0}$C for 30 minutes. 60µl of SDS and 3µl of Proteinase K was added and mixed and incubated at 55$^{0}$ C for 2 hours. After incubation, equal volume of Tris saturated phenol (900 µl) was added. The mixture was centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to a fresh tube and equal volume of PCI (Phenol: Chloroform: Isoamyl alcohol) was added. The extraction was repeated until no protein precipitate was obtained at interface. The aqueous phase was transferred to a fresh tube and added 0.1 volumes of 3M Sodium Acetate (pH 5.5). Mixed gently and added 2.5 volume of the ice cold Ethanol, mixed by inverting the tubes and incubated the mixture at –80$^{o}$C for 30 minutes. It was centrifuged at 10,000 rpm for 10 minutes and then discarded the supernatant. To pelleted DNA, equal volume of 70% ethanol was added and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded. The pellet was dried and dissolved in 100 µl of TE buffer. 20 µl of DNA was loaded in 1% Agarose gel.

**PCR amplification of 16S rRNA gene**

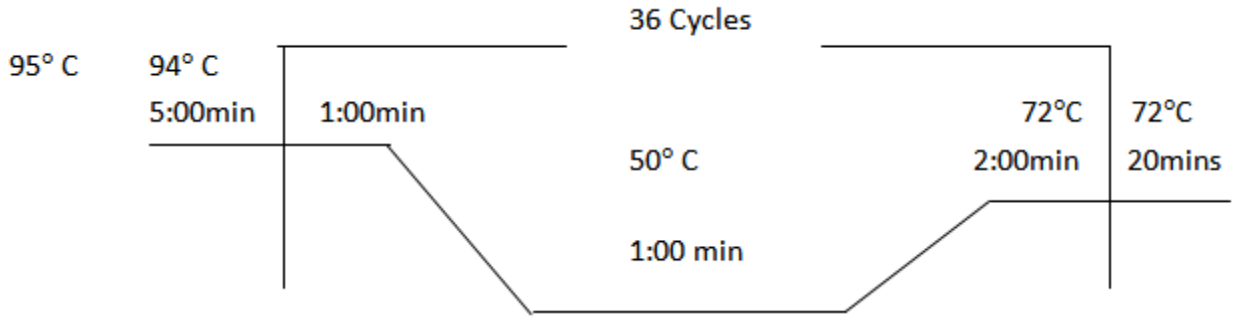
**TEST:**

- Total volume 50µl
- Genomic DNA =3µl
- 8F Primer =2.5µl
- 1490R Primer =2.5µl
- Bioron master mix =20µl
- Deionized water =22µl

**Control:** Total volume 50µl

- Genomic DNA =3µl
- Forward Primer =2.5µl
- Reverse Primer =2.5µl
- Deionized water =42µl

**Amplification cycle for 16S rRNA gene:**



**Separation of amplified PCR products:** 12 µl of amplified PCR product was loaded in the 1% Agarose gel electrophoresis.

**Plasmid isolation:** A single bacterial colony was transferred in to 5 ml of Bushnell Hass broth. The culture was inoculated over night at 37ºC with vigorous shaking. 1.5ml of the culture was poured in microcentrifuge tube and centrifuged at 5000rpm for 15 min at 4$^{o}$C. The supernatant was discarded and the pellet was resuspended in 100µl of ice cold solution I by vigorous vortexing. Then 200µl of freshly prepared solution II was added to the tube and it was tightly closed by inverting the tube 5 times and it was stored.

150µl of ice cold solution III was added to the tube and inverted to dispense solution and keep it undisturbed for 15 minutes. Then mixture was centrifuged at 10,000rpm for 5 min. The supernatant was mixed with double the volume of precipitation solution at room temperature and kept for few min. The pellet was washed with wash buffer. Then supernatant was removed carefully without disturbing pellet. The pellet was air dried and stored with 50µl of TE buffer. Then 7µl of loading dye was added and mixed well. 20µl of sample was taken and introduced into the well.

**Agarose gel electrophoresis of plasmid DNA:** The isolated plasmid was analyzed by agarose gel electrophoresis using 1.5% agarose gel in lX TE buffer and with 2µl of ethidium bromide was incorporated for plasmid DNA staining. 25µl of samples were added to each well and was run in lX TE buffer at 100V for 30 min. Hind III digested DNA was used as the molecular weight marker.

**Plasmid curing:** The role of plasmids in the bacteriocin production was confirmed by curing the plasmid with acridine orange at a concentration of 5 µg /ml which was added to the culture broth and incubated for 24h. The plasmid cured strains were screened for bacteriocin production.

**RESULTS:**

1. **Isolation of bacteria:** Creamy white, spherical shaped colonies were observed on MRS medium from the cheese and sub cultured for further analysis. Result was shown in **plate 1**.
2. **Biochemical characterization:** The biochemical characters of the isolates were studied, and are tabulated in **table 1 and figure 6-9**.
3. **Carbohydrate Fermentation Profiles:** In the experiment, samples are fermented to glucose, fructose, maltose, lactose, ribose and galactose. They are differing in their acid production and gas production. Results are shown in **table 2 and figure 10**.
4. **Antibiotic Susceptibility Test:** The antibiotic susceptibility study showed that the sample was highly was sensitive to amikacin. They are resistant to most antibiotics. Results are shown in **table 3 and plate 2.**
5. **Acidity Resistance Test:** The sample was found to be survived longer, especially in the range of 6 and 7 the results are shown in bar diagram.
6. **Bile Tolerance Test:** Tolerance to bile allows the strains to survive in the small intestine. This experimental sample was found to be having increase in the absorbance that indicates that it was more tolerant to bile in all concentration. The coefficient of growth inhibition in all concentration are calculated and tabulated in table 4 and bar diagram.
7. **Hemolytic activity:** With the help of hemolytic activity, it was identified as non pathogen.the result was shown in **plate 3.**
8. **Inhibitory activity:** LAB can produce antagonistic compound that vary in their spectra of activity. The selected *Lactobacillus* strain showed inhibitory against both gram positive and gram negative bacteria, and in addition to *Aspergillus flavus*. In disc diffusion and well diffusion methods, LAB shows strong inhibitory effect against *Pseudomonas* and *Serratia.* Diameter of inhibition zones are shown in table 5 and plate 4-5
9. **Partial purification and charaterization:** The effect of heat, storage time, pH , enzymes and surfactant on bacteriocin activity were determined using *Serratia* as indicator organism.

Bacteriocin produced by *L.plantarum* remained constant activity after heating at 90$^{o}$C for 10 min and not in 90 mins.the stability maintained for 60 min at 4$^{o}$C and -20$^{o}$C. The was no activity obtained in 37$^{o}$C. The pH remained stable from 2-6.

The proteolytic enzyme and uv did not affect the activity of bacteriocin produced by test isolate. Hexane and di ethyl ether did not result in removal bacteriocin produced at the aqueous phase to the organic phase, while choroform destroyed some bacteriocin activity.

However, when different alcohols such as n-propanol and iso-amylalcohol were used in the extraction procedure, bacteriocin was removed from the aqueous phase and removed from the organic phase. Exposure to surfactants resulted in an increase in the bacteriocin activity

Purification steps of the bacteriocin are summarized in **Table 7**. The bacteriocin of LAB recovered following the 60% saturation of the culture broths with ammonium sulphate with an increase to Total activity of 250000 (Fraction 1). The second step in the purification protocol was Trichloroacetic acid precipitation of Fraction 1. At this stage of purification, the total activity recovery was 2000. Results shown in **plate 6** and **in bar diagram**.

10. **Plasmid isolation and curing:** Plasmid isolated remained 12Kb. In plasmid curing the sample showed that bacteriocin was chromosomally mediated.the result was showed in **figure 13 and plate 7.**

11. **Genomic DNA isolation:** A discrete band was observed in 1% agarose gel on UV illumination after loading the Genomic DNA sample isolated. Shown in **figure 11**.

12. **PCR amplification of 16s rRNA gene:** The isolated DNA sample was amplified and occurrence of amplification was confirmed by agarose gel electrophoresis which was indicated in **figure 12** discrete bands having size of 1.5 Kb.

13. **Sequencing:** The PCR product was purified. The purified product was send for sequencing and the resultant sequences run in BLAST software in NCBI server.

**TABLE 1: BIOCHEMICAL CHARACTERISTIC OF THE LAB**

| | |
|---|---|
| **Gram stain** | + |
| **Morphology** | Rod |
| **Motility** | - |
| **Gas from glucose** | - |
| **Growth at,** | |
| **4$^{o}$C** | + |
| **15$^{o}$C** | + |
| **45$^{o}$C** | - |
| **Grown in** | + |
| **6.5%NaCl** | + |
| **7%NaCl** | - |
| **10%NaCl** | |
| **Catalase test** | + |
| **Methyl red test** | + |
| **Arginine hydralase test** | + |

**TABLE 2: CARBOHYDRATE FERMENTATION TEST**

| CARBOHYDRATES | LAB | |
|---|---|---|
| | ACID PRODUCTION | GAS PRODUCTION |
| Glucose | ++ | + |
| Lactose | ++ | - |
| Fructose | + | - |
| Ribose | + | - |
| Maltose | ++ | - |
| Arabinose | - | - |
| Rhamnose | - | - |
| Galactose | ++ | - |
| Mannitol | + | - |
| Starch | - | - |
| Glycogen | ++ | + |
| Sorbitol | + | - |
| Glycerol | - | - |

++ strong fermentation; + weak fermentation; - no acid or gas production

**TABLE 3: ANTIBIOTIC SUSCEPTIBILITY TEST-ZONE DIAMETER**

| ANTIBIOTICS | LAB |
|---|---|
| Ampicillin | **S** |
| Amikacin | **S** |
| Amoxycillin | **S** |
| Bacitracin | **S** |
| Cefazolin | **R** |
| Ceftazidime | **R** |
| Chloramphenicol | **S** |
| Citrofloxacin | **S** |
| Co-trimazole | **R** |
| Erythromycin | **S** |

| | |
|---|---|
| Kanamycin | **R** |
| Methicillin | **R** |
| Nalidixic acid | **R** |
| Streptomycin | **S** |
| Tetracyclin | **R** |

S- Sensitive R- Resistance

**TABLE 4: COEFFICIENT OF INHIBITION IN MEDIUM WITH BILE**

| The Oxgall Added (%) | Time In Hours | LAB (OD 600nm) |
|---|---|---|
| 0.1% | 2 Hrs | 0.058 |
| | 4 Hrs | 0.051 |
| | 6 Hrs | 0.046 |
| | 8 Hrs | 0.040 |
| 0.2% | 2 Hrs | 0.062 |
| | 4 Hrs | 0.054 |
| | 6 Hrs | 0.048 |
| | 8 Hrs | 0.042 |
| 0.3% | 2 Hrs | 0.069 |
| | 4 Hrs | 0.061 |
| | 6 Hrs | 0.056 |
| | 8 Hrs | 0.047 |
| 0.4% | 2 Hrs | 0.072 |
| | 4 Hrs | 0.062 |
| | 6 Hrs | 0.053 |
| | 8 Hrs | 0.044 |

Growth inhibition coefficient <0.5 – Bile tolerant species

**TABLE 5: MEASUREMENT OF INHIBITORY ACTIVITY**

| Indicator Organisms | Disc Diffusion Method Zone of Inhibition (mm) |
|---|---|
| **Gram-positive bacteria** | |
| *Staphylococcus aureus* | 14 |
| *Bacillus subtilis* | 12 |
| *Enterococcus* | 10 |
| **Gram-negative bacteria** | |
| *Pseudomonas aeruginosa* | 21 |
| *Escherichia coli* | 11 |
| *Shigella spp.* | 8 |
| *Samonella* | 8 |
| *Chromobacterium* | 14 |
| *Klebsiella* | 8 |
| *Serratia* | 18 |
| **Fungi** | |
| *Aspergillus flavus* | 12 |

**TABLE 6: EFFECT OF ORGANIC SOLVENTS ON BACTERIOCIN**

| Organic Solvents | LAB Zone of inhibition (mm) | |
|---|---|---|
| | Organic phase | Aqueous phase |
| I – amylalcohol | 10 | 6 |
| Choloform | 8 | 8 |
| N – propanol | 11 | 7 |
| Hexane | - | 16 |
| Di – ethylether | - | 18 |

Diameter of the well -6mm; Control = bacteriocin without addition of organic solvent

**TABLE 7: EFFECT OF pH ON BACTERIOCIN**

| pH | LAB Zone of Inhibition |
|---|---|
| 2 | 14 |
| 4 | 14 |
| 6 | 18 |
| 8 | 18 |
| 10 | 14 |
| 12 | 12 |

**TABLE 8:EFFECT OF STABILITY ON BACTERIOCIN**

| Treatments | LAB |
|---|---|
| Control | 24 |
| Tritox X – 100 | 18 |
| Tween 20 | 20 |
| Tween 80 | 20 |
| Sodium dodeyl sulphate | 16 |
| Lysozyme | 16 |
| Proteinase K | nd |
| UV light | 14 |

**TABLE 9: PURIFICATION OF BACTERIOCIN PRODUCED BY LAB**

| Organism | Purification Stages | Volume ml | Activity (Auml-) | Total activity (AV) a |
|---|---|---|---|---|
| LAB | Culture supernatant | 1000 | 5000 | 5000000 |
| | Ammonium sulphate precipitation (Fraction 1) | 25 | 10000 | 250000 |
| | Tricholoroacetic acid Precipitation (Fraction 2) | 1 | 2000 | 2000 |

**TABLE 10: FTIR**

| Absorption range frequency ($cm^{-1}$) | Functional groups | Mode of vibration |
|---|---|---|
| 3375 | Alcohol, phenol | Streching vibration |
| 3296 | Alkynes | Streching vibration |
| 2979 | Alkanes | Streching vibration |
| 2930 | Alkanes | Streching vibration |
| 2355 | Alkanes | Streching vibration |
| 2060 | Alkanes | Streching vibration |
| 1647 | Alkenes | Streching vibration |
| 1542 | Aromatic | Streching vibration |
| 1453 | Aromatic | Streching vibration |
| 1402 | Aromatic | Streching vibration |
| 1310 | Ether | Streching vibration |
| 1238 | Ether | Streching vibration |

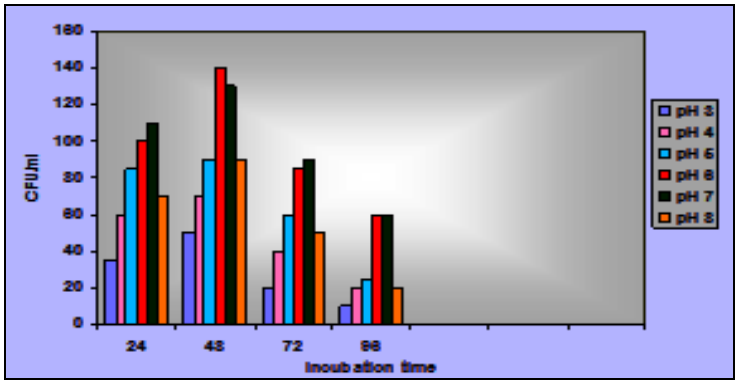



**FIGURE 1: SURVIVAL RATE OF LAB IN MRS MEDIUM AT DIFFERENT pH**

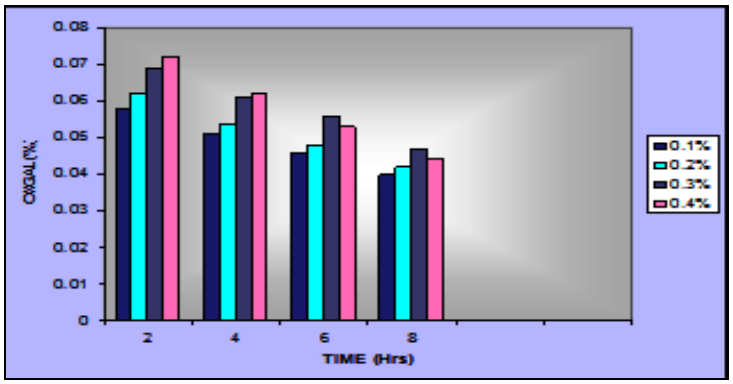



**FIGURE 2: BILE TOLERANCE**

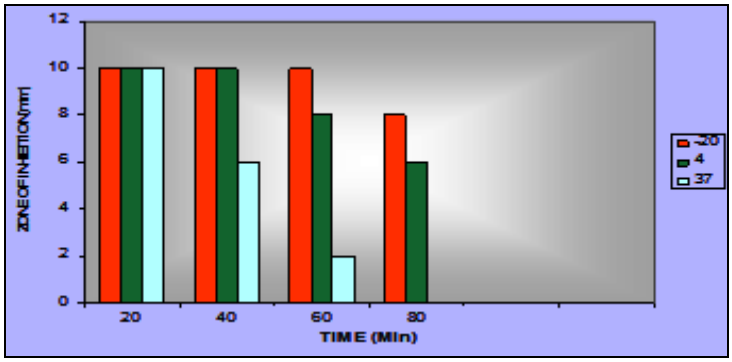



**FIGURE 3: EFFECT OF STORAGE**

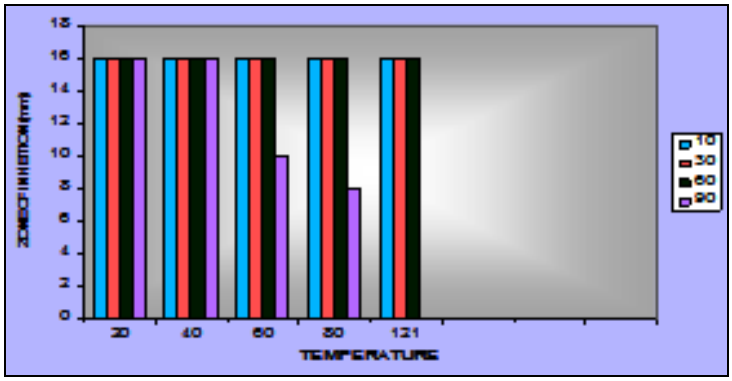



**FIGURE 4: EFFECT OF TEMPERATURE**

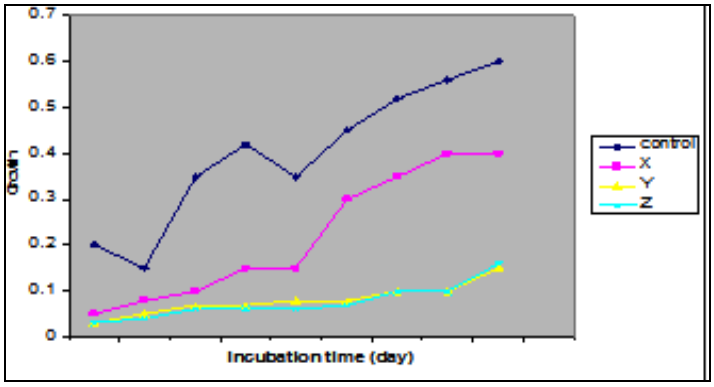



**FIGURE 5: SUPPRESSION OF *ASPERGILLUS FLAVUS***

X- *A.flavus* grown first before inoculated with LAB; Y- LAB grown first before inoculated with *A.flavus*; Z- Both *A.flavus* and LAB were inoculated at the same time

**PLATE 1: ISOLATED ORGANISM**

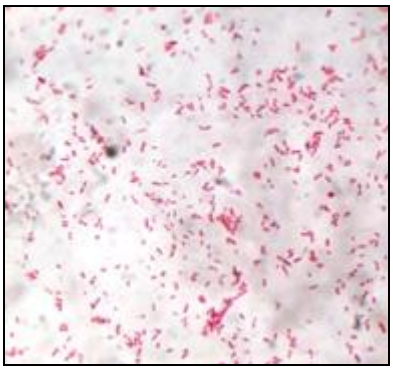


**FIGURE 6: GRAM STAINING**

**FIGURE 7: METHYL RED TEST**

**FIGURE 8: ARGININE HYDRALASE TEST**

**FIGURE 9: CATALASE TEST**

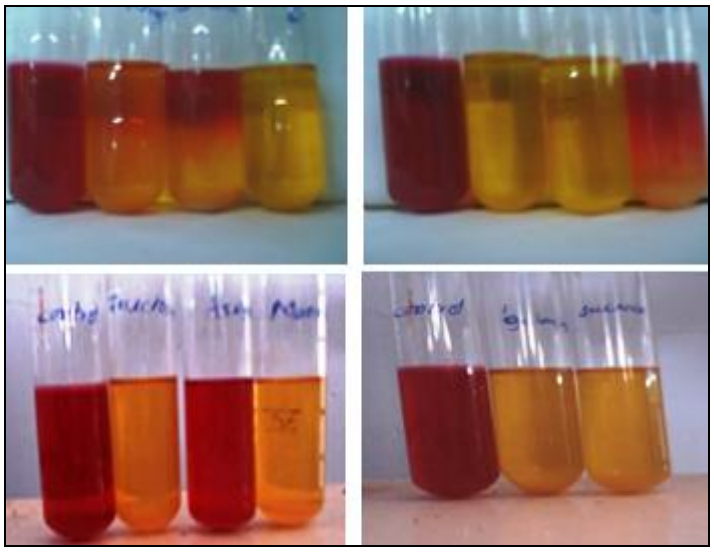


**FIGURE 10: CARBOHYDRATE FERMENTATION PROFILE**

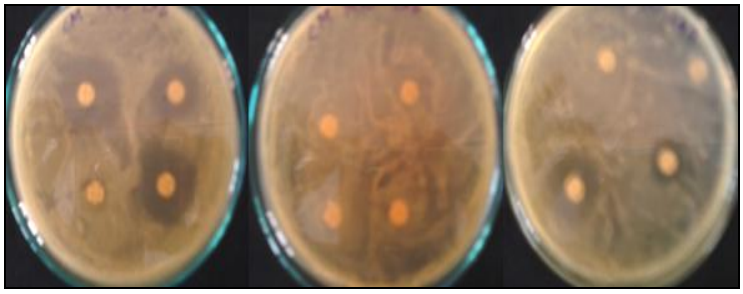


**PLATE 2: ANTIBIOTIC SUSEPTIBILITY TEST**

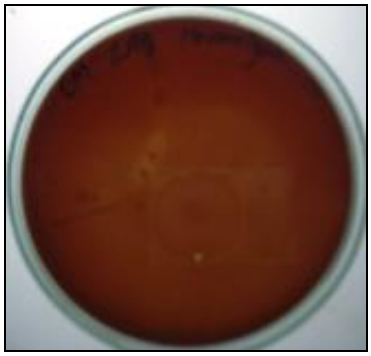
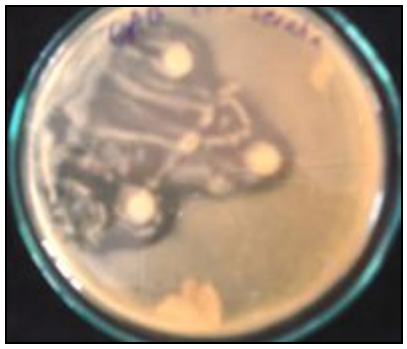
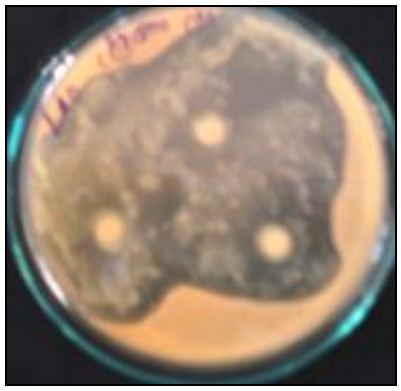


**PLATE 3: HEAMOLYTIC ACTIVITY**



**Zone of inhibition of *Chromobacterium violaceum***



**Zone of inhibition of *Serratia sp.***

**Zone of inhibition of *Pseudomonas Sp.***

**PLATE 4: ANTIBACTERIAL ACTIVITY (DISC DIFFUSION METHOD)**

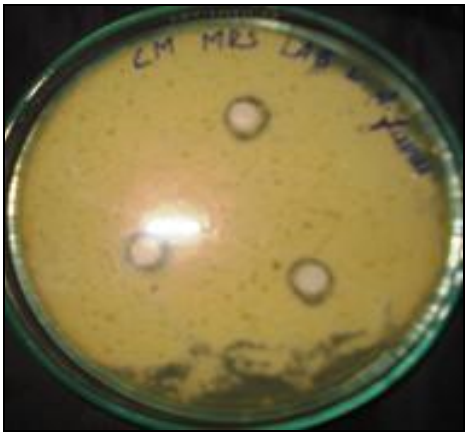


**PLATE 5: ANTIFUNGAL ACTIVITY**

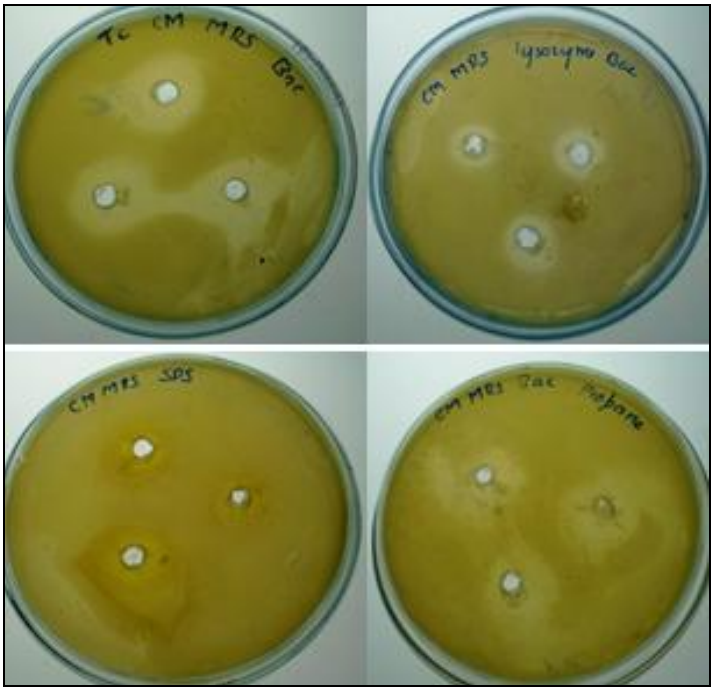


**PLATE 6: EXTRACTION OF BACTERIOCIN WITH ORGANIC SOLVENTS**

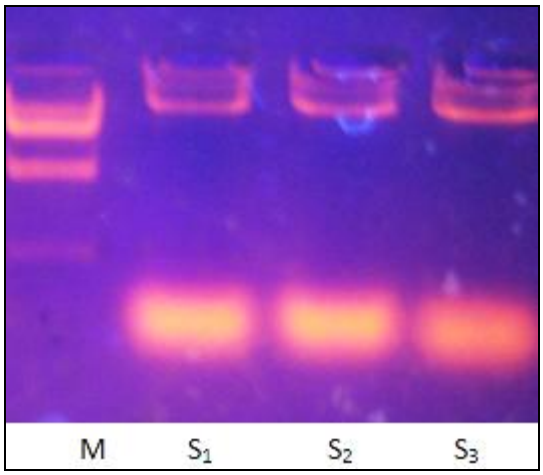



**FIGURE 11: ISOLATED GENOMIC DNA**

(M= Marker, $S_1$, $S_2$, $S_3$ samples)

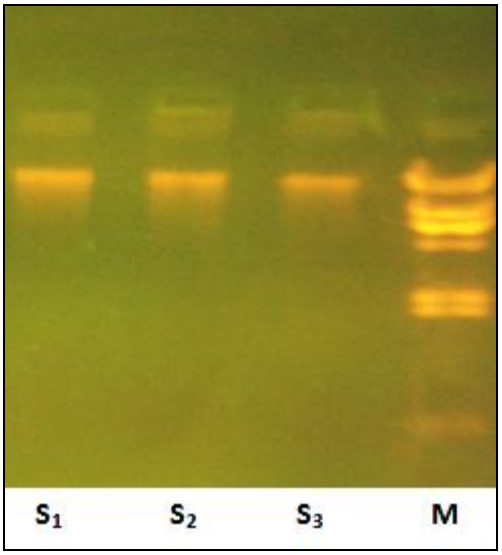



**FIGURE 12: PCR AMPLIFIED PRODUCTS OF 16S rRNA GENE**

(M= Marker, $S_1$, $S_2$, $S_3$ samples)

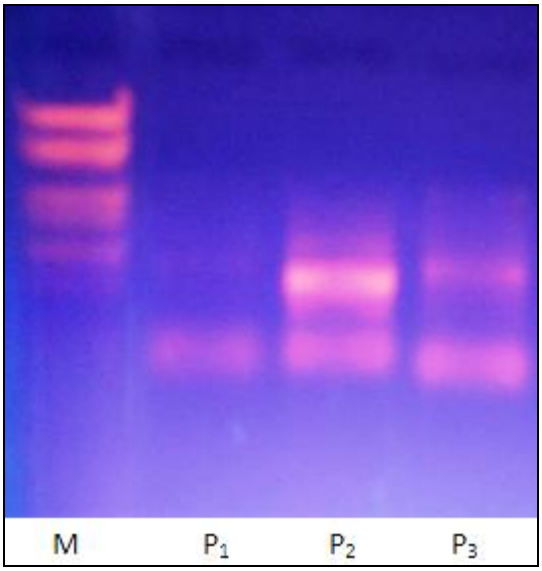



**FIGURE 12: PLASMID PROFILING**

(M= Marker, $P_1$, $P_2$, $P_3$ Samples)

**PLATE 8: PLASMID CURING**

**DISCUSSION:** The isolated experimental sample was biochemically identified as Gram positive, catalase positive rods, non motile, methyl red positive, which phenotypically related to genus of *Lactobacillus*. Many *Lactobacillus* are resistant to antibiotics. This resistance attributes are often intrinsic and non transmissible. Intrinsically antibiotic resistance probiotic strains may benefit patients whose normal intestinal microbiota has become unbalanced or greatly reduce in numbers due to the administration of various antimicrobial agents [10].

Our experimental sample are also resistant to most of the antibiotics. So sample may be benefited for patients to balance the administration of antibiotics. The resistance of bacteria to antibiotics depends on their genus, species, strain and phase of logarithmic growth. Moreover, antibiotic therapy may cause diarrhea of varying course. The pathogenesis of the development of this type of diarrhea is associated with quantitative and qualitative changes in the composition of the intestinal micro flora. The application of probiotics which restore the intestinal homeostasis is widely accepted as a safe alternative of antibiotic therapy.

*L. acidophilus* bacteria as one of the species commonly accepted as probiotics turned out quite effective in preventing aliments caused by the application of Ampicillin, neomycin and amoxicillin.

Our experimental sample can be used as probiotic to treat antibiotic associated diarrhea. As it was resistant to cefazolin, cefazidime, kanamycin, tetracyclene, methicillin and nalcidic acid.

Some antimicriobial metabolite like diacetyl inhibited Gram negative bacteria more than Gram positive bacteria[11].All the pathogens were inhibited by *L. plantarum* strains with *Staphylococcus aureus* having the highest inhibitory zone followed by E. coli and *S. typhii*. [12], *L. plantarum* had a broad antimicrobial inhibitory spectrum, with activity against several strains of bacterial pathogens.the present study also, in concordance with the above reported as *P. aeroginosa and Serratia* are the most sensitive bacteria. CFS of *Lactobacillus* gave zones of inhibition onto the pathogenic strains. This indicate that *Lactobacilli* are capable of synthesysing inhibitive substances of pathogenic bacteria, it can be a protein. This inhibitive substances produced by *Lactobacillus* strain act differently, they may be either bacteriostatic or bacteriocidal. The inhibitory activity of our expeimental sample was identified by the disc difffusion and well diffusion method, the supernatant of *Lactobacillus* were used and pH of the supernatant was adjusted to 7.0. So the inhibition zone was seeen around disc and well may be dependent on bacteriocins activity [13].

Probiotics cultures must survive in the environment with gastric and bile acids, when viable cells go through the gastrointestinal tract. Resisting at pH 3.0 and growing in the medium containing 0.1% bile acids are considered as standards for acid and bile tolerance of probiotics culture . The experimental sample survived at pH 6 and also the medium containing 1% bile. The experiment shows higher resistance of the sample *Lactobacillus* to bile concentration corresponding to the concentration prevailing in the human gastro-intestinal tract.

The selective plating method was suitable for the identification of specific microorganism present in the samples. The results strengthen the rising opinion that selective media should be evaluated for the specific strains of species of interest in particular samples . The effect of heat, storage time, pH, enzymes and surfactant on bacteriocin activity were determined using *E. faecalis* as indicator organism. The inhibitory compound produced by the test isolate was considered to be heat stable [8]. In our experimental sample *Serratia* was used as indicator organism.

The pH remained stable from 2-6. In the other hand the bacteriocins were shown to be stable over a broad pH range with all peptides maintaining some antimicrobial activity within the pH range of pH 3 to 10. Bacteriocins differ greatly with respect to sensitivity to pH (Abdelbasse *et al*., 2008). In our experimental sample the pH remained stable at 6-7 [14]. Complete inactivation or significant reduction in antimicrobial activity was observed after treatment of the cell-free supernatant with Proteinase K, pronase and trypsin [15]. In concordance to above the proteinase K inactivated the bacteriocin activity, but lysozyme showed zone.

The proteolytic enzyme and uv did not affect the activity of bacteriocin produced by test isolate. In addition to the broad inhibition spectrum, its technological properties and especially heat and storage stability, indicate that plantaricin AA135 has potential for application as a biopreservative to control pathogens in processed foods [16]. Bacteriocin produced by our experimental sample remained constant activity after heating at 90$^{o}$C for 10 min and not in 90 mins. The stability maintained for 60 min at 4$^{o}$C and -20$^{o}$C. The is no activity obtained in 37$^{o}$C.

Plantaricin AA135 was not removed from the aqueous phase with very apolar solvents such as hexane and di-ethyl ether. Inhibitory activity of the bacteriocin was almost completely destroyed by chloroform. However, when various alcohols were used in the extraction method, plantaricin AA135 was removed from the aqueous phase and could be recovered from the organic phase. Butanol extraction exhibited complete recovery of plantaricin AA135 activity suggesting that at least part of the plantaricin AA135 molecule has a hydrophobic character [16].

Hexane and diethyl ether did not result in removal bacteriocin produced at the aqueous phase to the organic phase, while choroform destroyed some bacteriocin activity. However, when different alcohols such as n-propanol and iso-amylalcohol were used in the extraction procedure, bacteriocin was removed from the aqueous phase and removed from the organic phase. Exposure to surfactants resulted in an increase in the bacteriocin activity. Plasmid was isolated as 10Kb and curing shows that bacteriocin was chromosomally media.

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