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Original Research Article

Optimization of growth parameters for Bacteriocin producing *Lactobacillus* strain and its molecular characterization by RAPD-PCR

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Keywords

Bacteriocins; Lantibiotics; Lactobacillus; Antimicrobial Activity; RAPD-PCR.

A B S T R A C T

Lactobacillus strains isolated from four different sources like home made curd, pickle, olive and butter milk, were identified by morphological and biochemical characteristics. These strains were showing negative results to Catalase Test and Gas formation but were giving positive results to Lactose fermentation test. Antimicrobial assays were performed for all strains. Crude protein extract was further processed through column (Size exclusion) chromatography and SDS-PAGE. Strains were found to produce Bacteriocins with approximate molecular weight of 140kDa equal to Bacitracin which was used as standard. Media composition w. r. t. Glucose (Carbon source) and Peptone (Nitrogen Source) and growth parameters as pH and Temperature were optimized for strains from Buttermilk samples as they were showing highest antimicrobial activity. Pure cultures of these isolates were characterized by RAPD marker analysis. For RAPD analysis by PCR technique, 3 arbitrary primers belonging to operon series were selected. D18, D19 and D20 wee the Primers used for RAPD-PCR amplification. Dendrogram analysis for frequency similarity was done by using Alpha Imager Software and similarity matrix was generated.

Introduction

Food processing still has the main objective of providing a safe nutritious diet in order to maintain health. Maintaining or creating nutritional value, texture and flavour is an important aspect of food preservation, although, historically, some methods drastically altered the character of the food being preserved. The consumption of more food that has been formulated with chemical preservatives has also increased consumer concern and created a demand for more natural and minimally processed food. As a result, there has been a great interest in naturally produced antimicrobial agents. Jennifer Cleveland *et al* (2001) The need for the Industry to increase shelf life and provide safety to the food products have created huge interest in researchers to find natural and safe preservatives which can not only maintain the texture and quality

but also can act as antimicrobial agents against unwanted food pathogens as well. Food Preservation is a continuous war against the microorganisms spoiling the food or making it unsafe causing various infections reported by Mohana srinivasan *et al* (2012). The rapid rise and spread of multi-resistant bacterial pathogens have forced the consideration of alternative methods of combating infection. One of the limitations of using broad-spectrum antibiotics is that they kill almost any bacterial species not specifically resistant to the drug. Current solutions to this dilemma involve developing a more rationale approach to antibiotic use, which involves curtailing the prescription of drugs for anything other than bacterial infections, cycling through different drugs over a shorter time frame. Bacteriocins provide an alternative solution. With their relatively narrow spectrum of killing activity, they can be considered "designer drugs," which target specific bacterial pathogens. The development and use of such narrow-spectrum antimicrobials not only increases the number of drugs on the pharmaceutical shelf but, more importantly, extends their shelf life. Bacteriocins were originally defined as the proteinaceous compounds that kill closely related bacteria Tagg *et al* (1976) in Maja Tolinački *et al* (2010) Bacteriocins are antibacterial proteins produced by bacteria that kill or inhibit the growth of other bacteria. According to *Klaenhammer*, 99% of all bacteria may make at least one bacteriocin and the only reason we haven't isolated more is that few researchers have looked for them. Many lactic acid bacteria (LAB) produce a high diversity of different bacteriocins. *Lactobacillus* bacteriocins are grouped as class-I bacteriocins (Lantibiotics), class-II bacteriocins (heats table, non-Lantibiotics), class-III (heat-labile proteins with large molecular mass) and class-IV (hydrophobic and heat-stable proteins, associated with lipids or carbohydrates). BACTIBASE is an open-access database designed for characterization of bacteriocins, and the number of entries continues to grow. The database contains 200 Bacteriocin sequences and most of which are products of gram-positive bacteria, particularly Lactic Acid Bacteria (LAB) Hammami *et al* (2010). As such they have become attractive candidates for use in food preservation (by inhibiting pathogens that cause food spoilage) and the pharmaceutical industry (to prevent or fight infections in humans or animals). The only bacteriocins currently employed in food preservation are those produced by LAB used in the production of fermented foods. Because LAB has been used for centuries to ferment foods, they enjoy GRAS (generally regarded as safe) status by the U.S. Food and Drug Administration (FDA). This permits their use in fermented foods without additional regulatory approval Margaret A. *et al* (2002). The purpose of this study was to analyze and enhance Bacteriocin production by the natural isolate of *Lactobacillus spp*. which we have tested for their antimicrobial activity, especially against spoilage microorganisms and its Molecular characterization by RAPD-PCR technique.

Materials and Methods

Isolation and Identification

Lactobacillus strains were isolated from four different sources like home made curd, pickle, olive, butter milk. The samples were serially diluted and were plated on MRS agar plates and incubated at 37ºC for 24 – 48 hours. Colony Characterization and Differential staining techniques (Gram's staining, Endospore

staining and Acid fast Staining) were performed along with various biochemical tests for identification of *Lactobacillus spp.*, according to Bergey's Manual. Antibacterial property was assayed by agar well method using different *Lactobacillus* cultures grown for 48 hrs in MRS Broth. Optimization of Media was done with respect to pH, Temperature, Carbon source (Glucose) and Nitrogen Source (Peptone). Screening of highest Bacteriocin producing strain/culture was done by agar well diffusion methods to observe clear zones.

Mass production, Purification and Assay

Mass Production of Bacteriocin by *Lactobacillus* was carried out by preparing growth media with optimized pH, Temperature, Carbon and Nitrogen source values. Production Media (1litre) was inoculated with 3% of 30ml inoculum culture in Fermentor of 3litre capacity and production was carried for 48-72 hours. 100ml of the Fermented broth was centrifuged at 5000rpm for 15minutes at 4˚C. The supernatant was used as crude enzyme extract for Ammonium Sulphate Precipitation using the principle of Salting-out of Proteins by standard procedures. The precipitate pellet was dissolved in and subjected to Dialysis. 1ml of the enzyme precipitate obtained from 80% saturated ammonium sulfate was dissolved in 20mM Phosphate buffer and were then kept for dialysis in 500ml of 20mM Phosphate buffer (pH-7.5) with continuous stirring. The buffer was changed 4 times at regular interval of 2 hours. Dialyzed concentrate of Enzyme was used as sample to load on to Gel Filtration column containing Sephadex G-100 Resin equilibrated with 20mM Phosphate buffer, pH-7.5. Gel Filtration was carried out at 1-1.5 ml / min flow rate using 20 ml of 20mM Phosphate buffer (pH-7.5) to wash column and fractions were collected (1-2 ml each). A sample of the purified enzyme was saved for later analysis. The fractions with the highest absorbance at 280 nm were taken for SDS PAGE using 10% resolving gel and 5% stacking Gel with pH 8.8 and pH6.8 respectively. Purified extract of protein was heated at 100°C for 5 minutes with 1X SDS gel loading buffer. Electrophoresis gel was stained using CBB dye with shaking at RT for 30-60 minutes.

Molecular characterization of *lactobacillus spp*.

Lactobacilli cells from 48hrs MRS broth culture were centrifuged at 6000rpm for 10mins to pellet out cells for DNA Isolation according to protocol given in Sambrook and Russell, Molecular Cloning, Third Edition, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York, 2001. DNA was isolated by using Phenol Chloroform and chilled Ethanol was used for precipitation of DNA. Dissolve the DNA pellet in 50µl TE buffer and store at 4°C. For quantitative estimation of DNA concentration, Optical Density (OD) at 260 nm is measured. DNA bands were observed by running DNA samples along with loading buffer in 1X Agarose gel prepared in 50X TAE Buffer. Electrophoresis was run using 1X TAE Buffer at 50V and 75mA till the dye reaches $3/4^{th}$ of the Gel. After running, Bands in the Gel was observed in UV.

DNA was amplified by RAPD-Polymerase Chain Reaction according to table given below: Most PCR methods can amplify DNA fragments of up to ~10 kilo base pairs (kb), although some techniques allow

for amplification of fragments up to 40 kb in size.

The amplified products were resolved by electrophoresis in 2% agarose gel using 1X TAE buffer at 60 volt for 2-5 hours. Gel was visualized by staining with ethidium bromide (1 µl/10 ml) and banding patterns were photographed over UV light using Gel Doc Instrument. All the other procedure for Agarose gel electrophoresis was done as stated in Electrophoresis of DNA point.

Molecular analysis

Phylogenetic variations were determined by converting RAPD data into a frequency similarity and analysed by unweighted pair method with arithmetic mean (UPGMA) cluster analysis to produce phylogenetic tree and Dendrogram analysis by using gel documentation system (Alpha Imager, Germany)

Results and Discussion

Four natural sources have been used for isolation of *Lactobacillus spp.* In isolation, Butter milk sample has showed maximum number of colonies with 11 CFU/plate and minimum of 3 CFU/plate with sample fermented olive.

The entire organisms showed negative result for Catalase, lactose Fermentation and Gas production but were showing positive results for as production. The culture from the Butter milk has shown the maximum diameter of clear zone of inhibition against *Bacillus subtilis.*

pH: The species from Butter milk sample showed the maximum diameter of zone of inhibition for pH 5.5 against *P. aeruginosa.*

Temperature: The species from Butter milk sample showed the maximum diameter of zone of inhibition at 37^{o}C against *P. aeruginosa.*

Glucose: The organism showing maximum zone of inhibition against *P aeruginosa* for 2% of Glucose concentration

Nitrogen: The organism is showing maximum zone of inhibition against *P. aeruginosa* for 1% concentration of Nitrogen.

Lactobacillus spp., was isolated from four different natural sources. The plates of 10^{-5} have been chosen for colony characteristics. The numbers of colonies for the respective samples were 7,11,3, & 6 respectively from each plate. It can be concluded that the highest number of colonies were found in case of butter milk culture and the least were found with that of olive culture. From the plates eleven colonies were selected resembling *Lactobacillus* colonies. *Lactobacillus* was thereafter confirmed according to Bergey's manual. Differential staining was carried out for the strains and results were recorded.

For the screening of Bacteriocin production all the four isolates were inoculated against pathogen *E.coli, Pseudomonas, Bacillus, Staphylococcus* on MHA medium, and out of that butter milk culture showed the maximum inhibition zone of 2.0cm against *Bacillus subtilis* (*Figure 2*).For further optimization, butter milk culture was selected. In order to optimize the condition for maximum production of Bacteriocin-pH, temperature, concentration of glucose and concentration of nitrogen has been varied in MRS media. The optimized pH

Fig.1 Buttermilk sample is showing the maximum no. of colonies

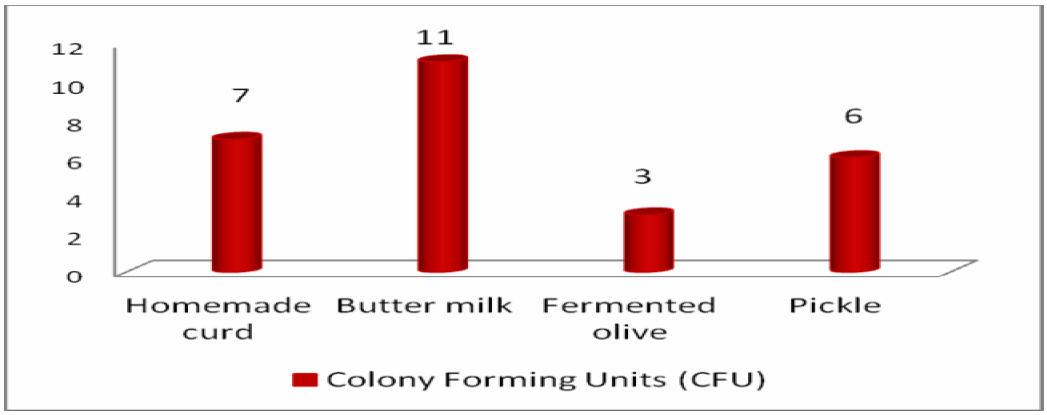

Fig.2 Zone of Inhibitions by different strains in different cultures

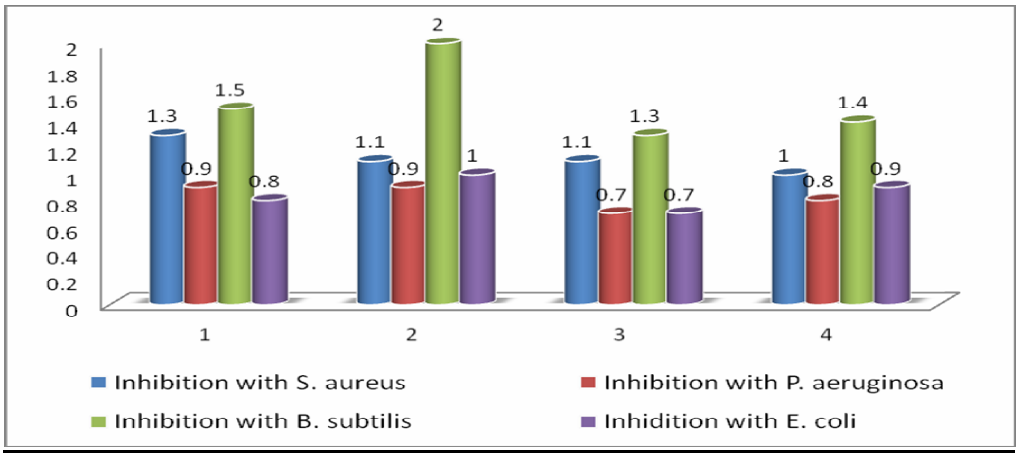

Fig.3 Zones of inhibition against different pH of Media

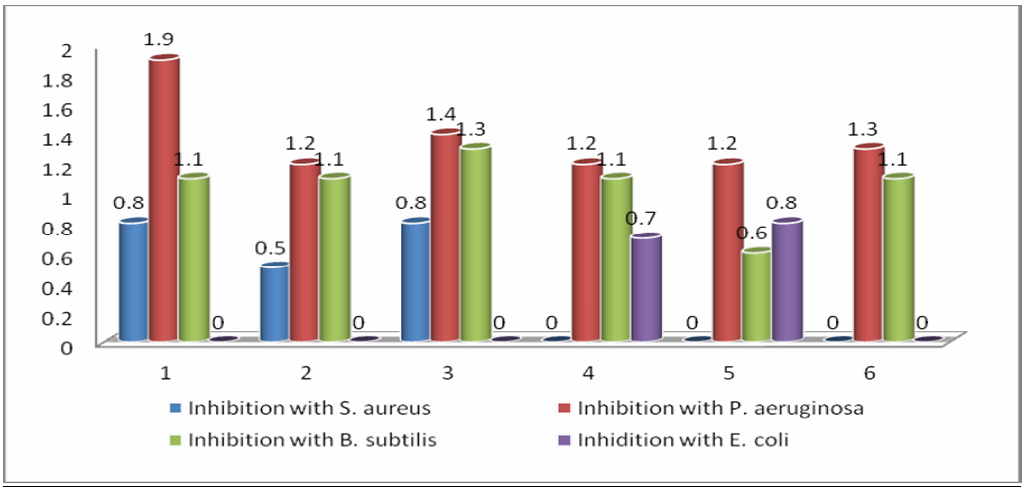

Fig.4 Zones of inhibition at different temperatures

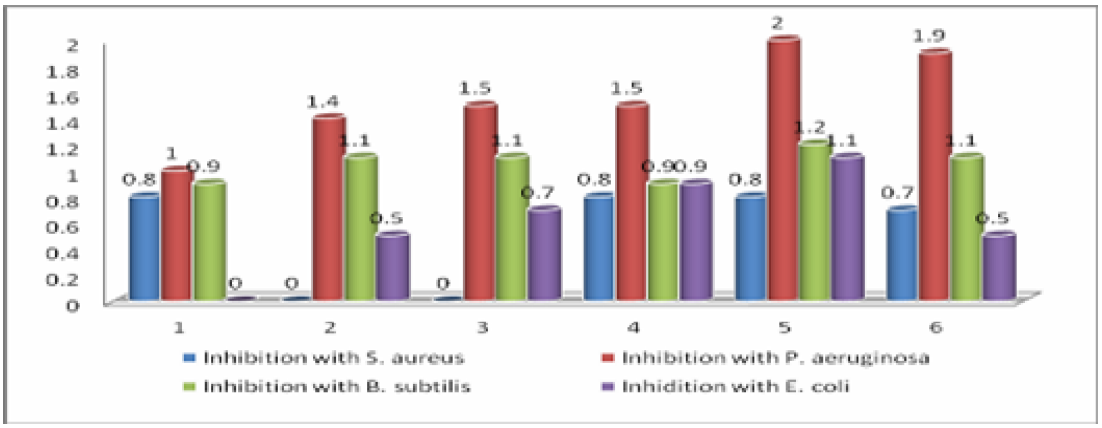

Fig.5 Zones of inhibition Different glucose concentration

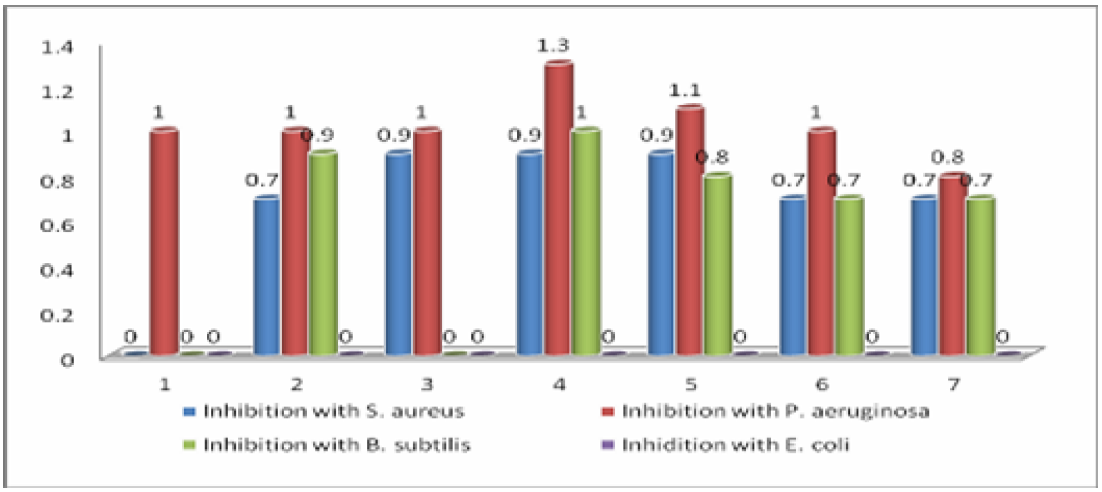

Fig.6 Zones of Inhibition by strains with different Nitrogen sources

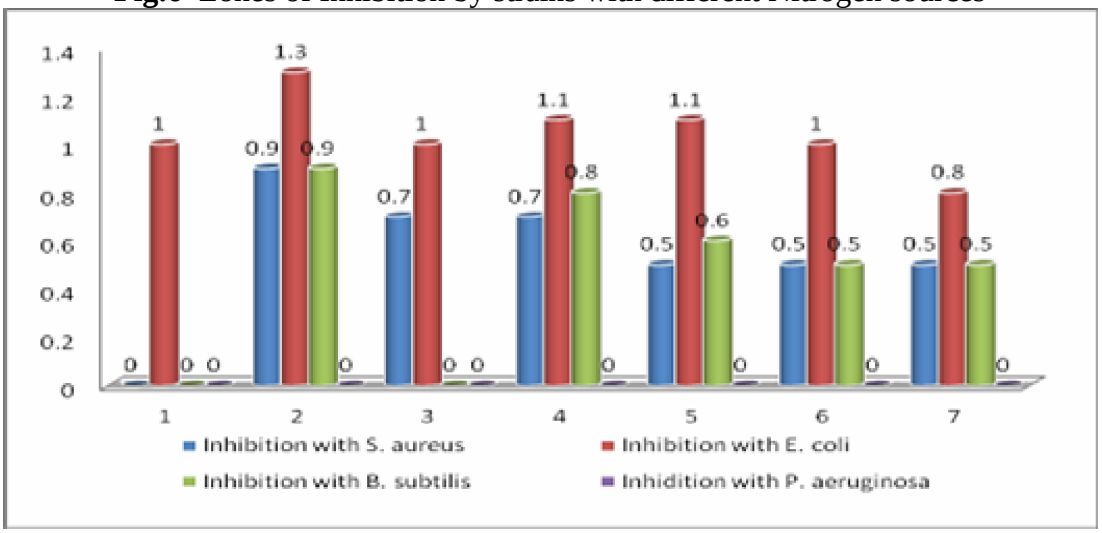

Fig.7 Graph for Optical Density of extracted fractions of protein. X-axis= No. of fractions. Y-axis= optical density.

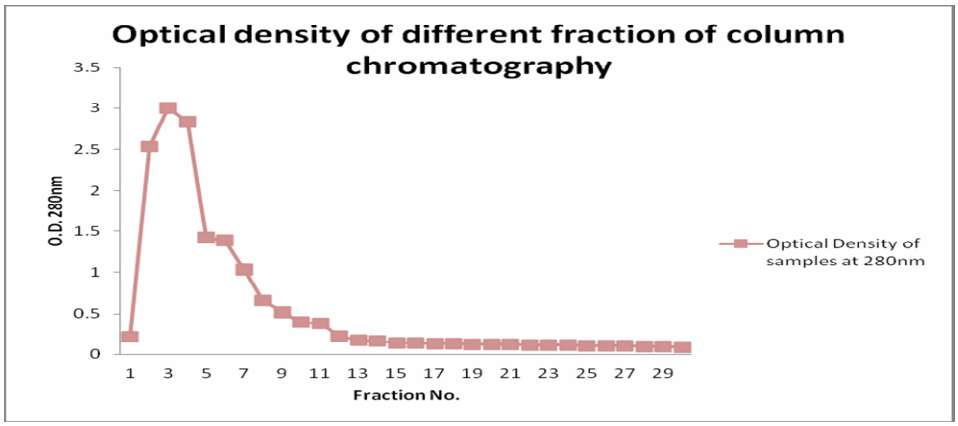

Fig.8 1= fraction no. 2, 2= fraction no. 4, 3= fraction no. 7, 4= fraction no. 11, 5= fraction no.15, M= marker Std = Standard Bacteriocin (Bacitraicin).

Molecular characterization of *Lactobacillus spp*:

Fig.9 1 = Homemade curd, 2 = Butter milk, 3 = Olive, 4 = Pickle.

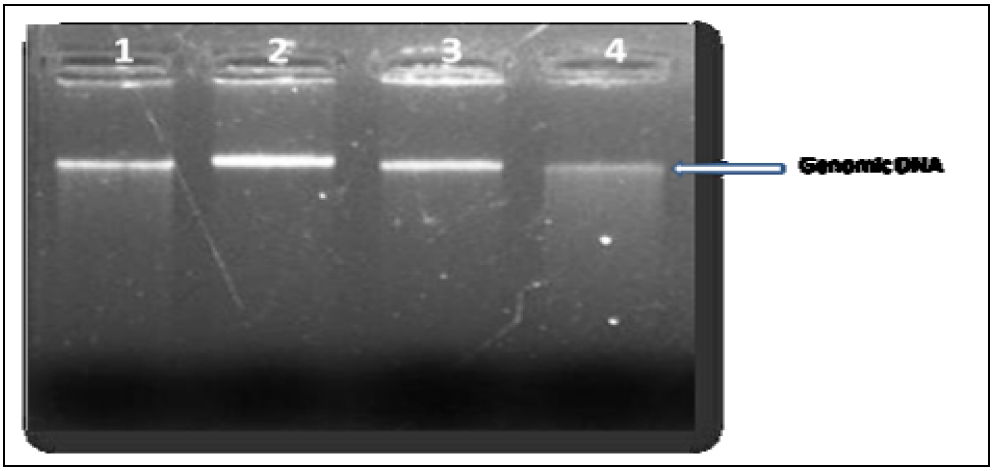

Fig.10 Quantification of four DNA (ng/µl) samples by Nanodrop
(a)= Homemade curd, (b) = Butter milk, (c)= Olive, (d)= Pickle

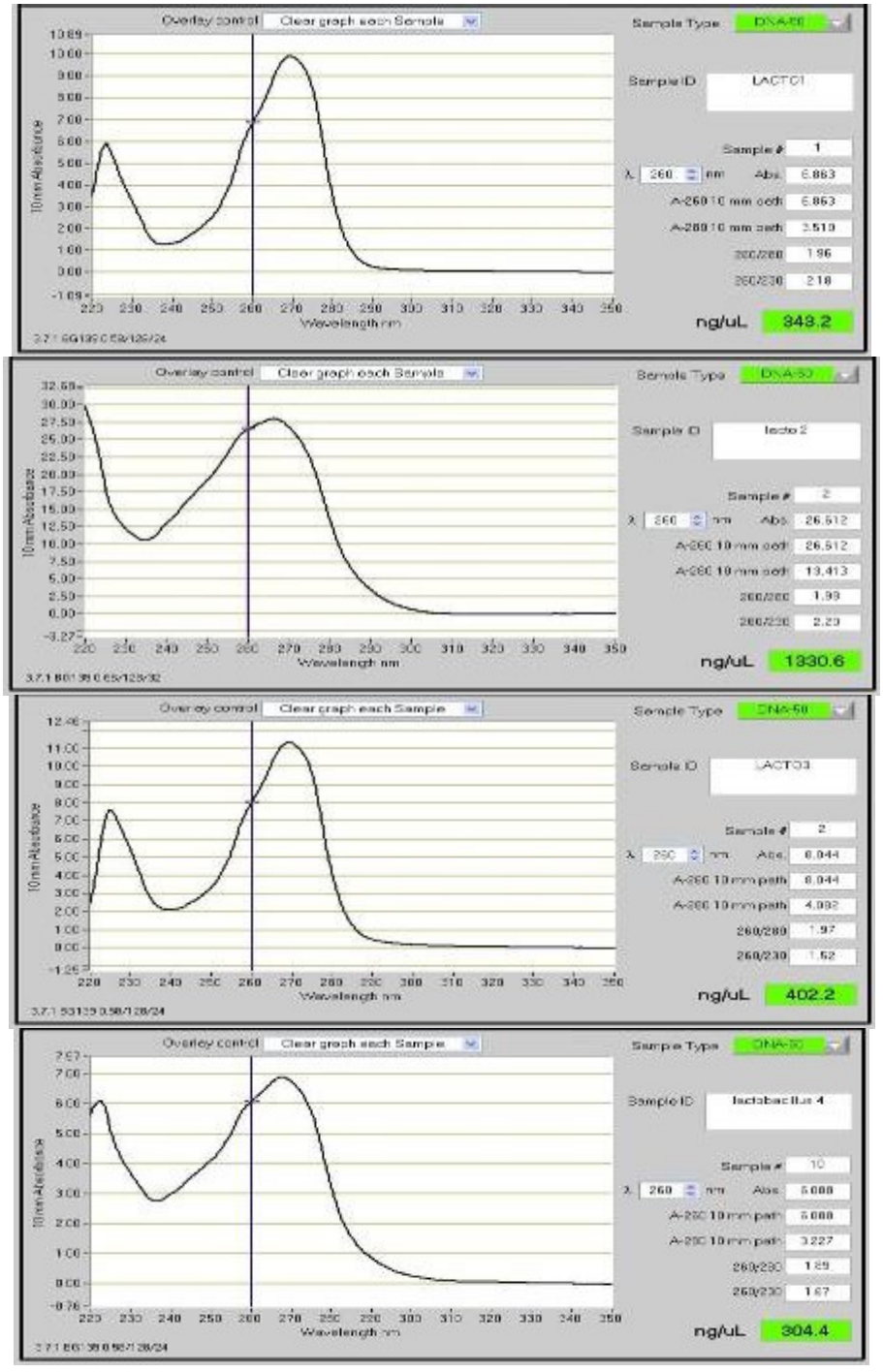

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Fig.11 Amplification with Primer D18

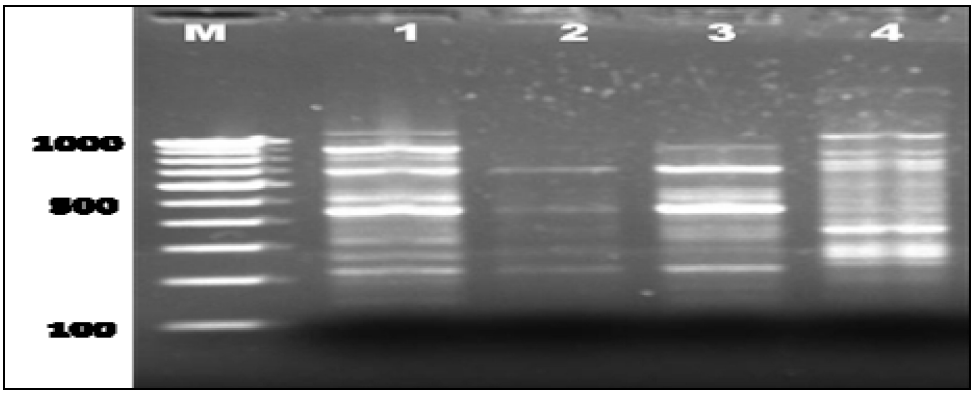

Fig.12 Dendrogram of Primer D18

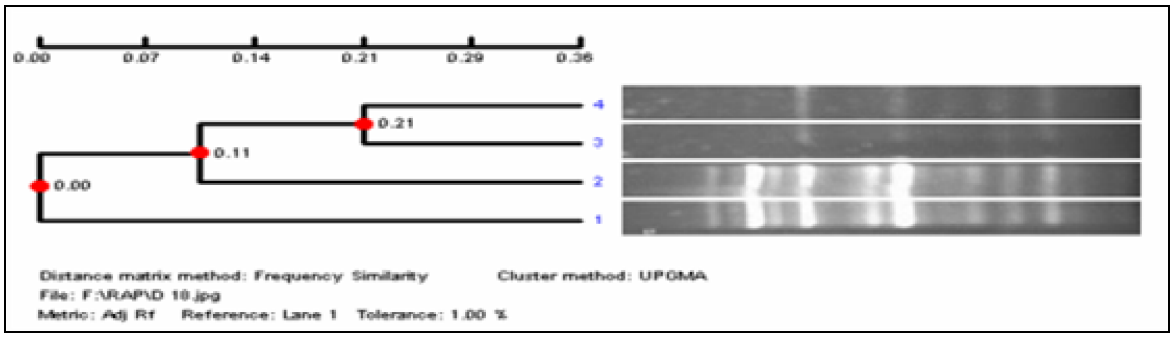

Table.1 Frequency similarity chart following Dendrogram of primer D18

Sl. No.	1st Sample	2nd Sample	3rd Sample	4th Sample
1st Sample	100.00	28.57	35.71	21.43
2nd Sample	28.57	100.00	50.00	50.00
3rd Sample	35.71	50.00	100.00	71.43
4th Sample	21.43	50.00	71.43	100.00

Fig.13 Amplification with Primer D19

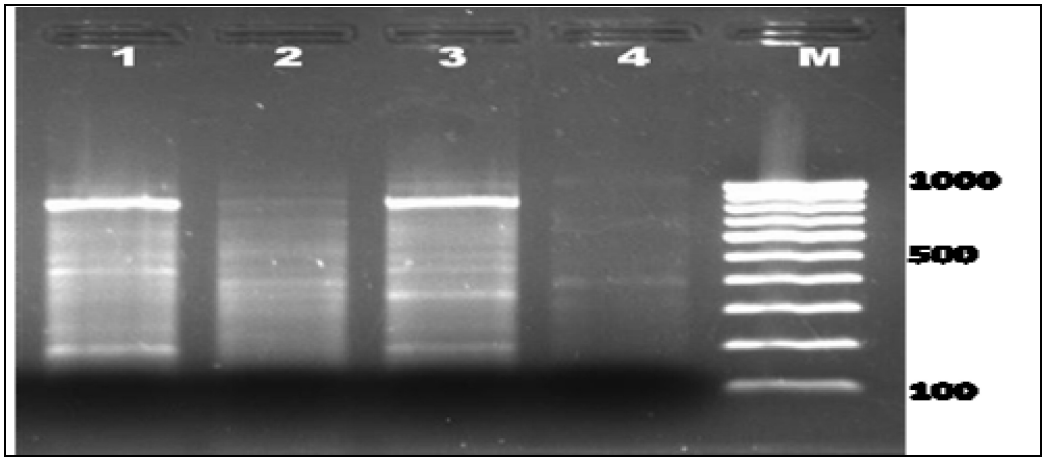

Fig.14 Dendrogram of Primer D19

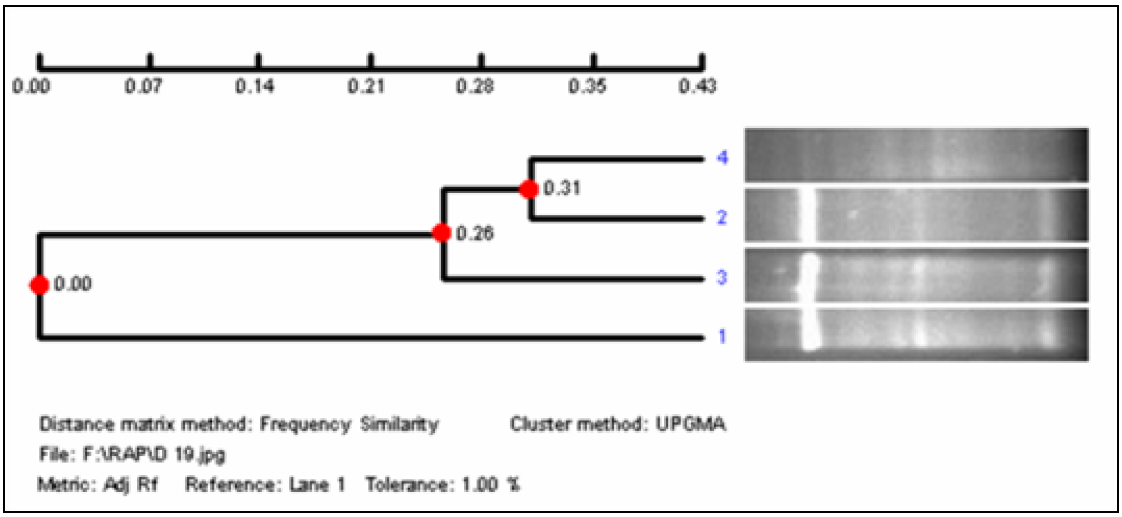

Table.2 Frequency similarity chart following Dendrogram of primer D19.

Sl. No.	1st Sample	2nd Sample	3rd Sample	4th Sample
1st Sample	100.00	11.11	22.22	11.11
2nd Sample	11.11	100.00	66.67	77.78
3rd Sample	22.22	66.67	100.00	66.67
4th Sample	11.11	77.78	66.67	100.00

Fig.15 Amplification with Primer D20

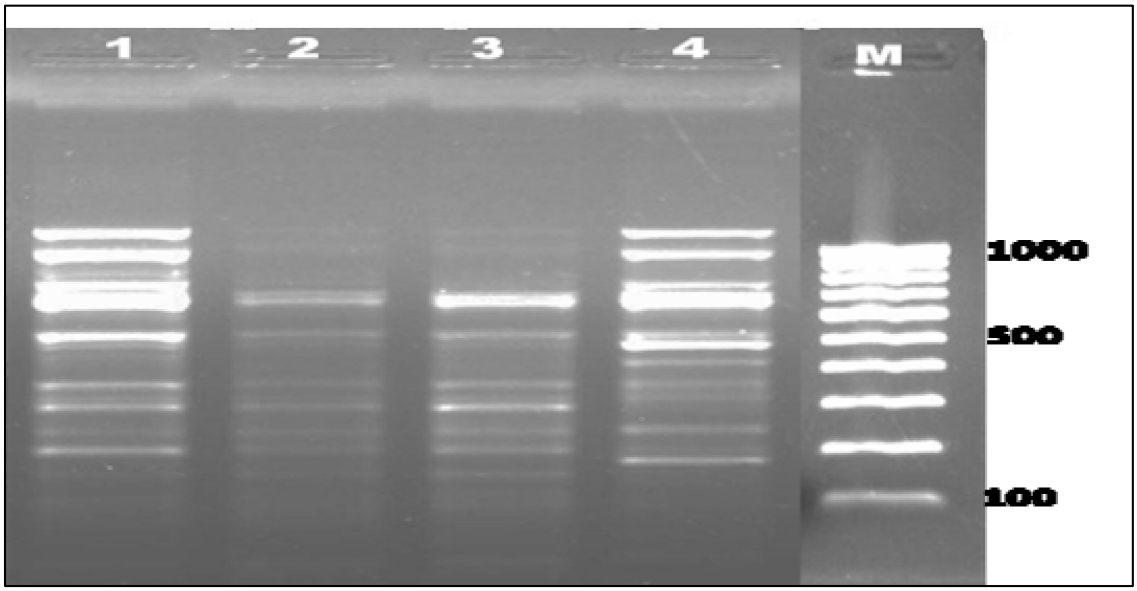

Fig.16 Dendrogram Primer D20

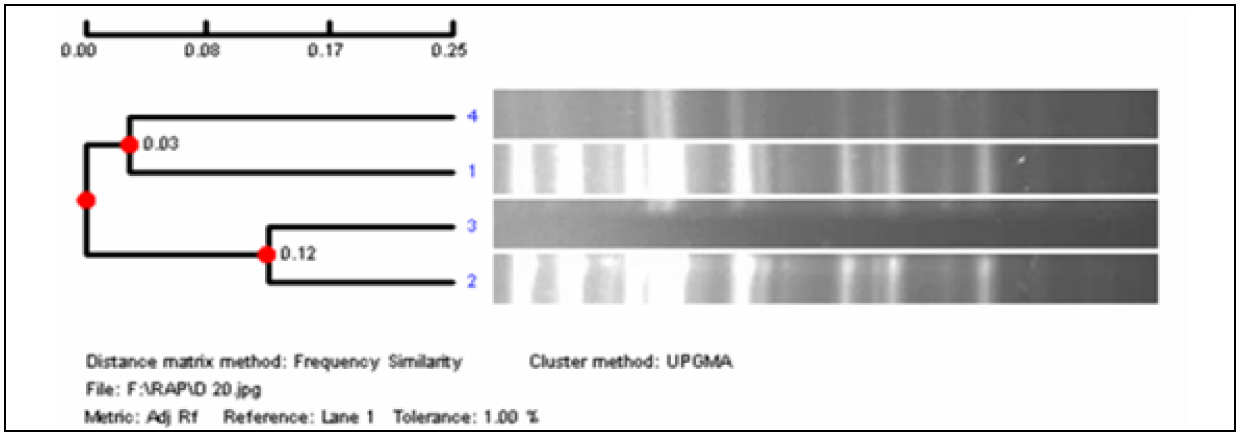

Table.3 Frequency similarity chart following Dendrogram of primer D20

Sl. No.	1st Sample	2nd Sample	3rd Sample	4th Sample
1st Sample	100.00	43.75	68.75	56.25
2nd Sample	43.75	100.00	75.00	37.50
3rd Sample	68.75	75.00	100.00	50.00
4th Sample	56.25	37.50	50.00	100.00

was 5.5 against *P.aeruginosa* for maximum production of Bacteriocin (Figure 3).The optimized temperature was 37□C (Figure 4) with 2% glucose (Figure 5), 1% peptone (Figure 6) against the same.

Bacteriocin was purified by ammonium sulphate precipitation method, dialysis and gel exclusion chromatography. 30 fractions were obtained from chromatography, among these 5 fractions (2, 4, 7, 11 & 15) were selected to perform SDS-PAGE. Bacitracin was taken as the standard for molecular weight determination of unknown protein. The molecular weight of Bacitracin is approximately 140KD. The bands of the unknown proteins coincided with that of standard Bacitracin (Figure 8).

The crude DNA, after running on the agarose gel, confirmed the presence of DNA but with some impurities. After purification, the samples were once again run on 1% agarose gel and the purity was confirmed. The Quality analysis and quantification of the obtained DNA samples was done using NanoDrop-spectrophotometer analysis which confirmed that the DNA obtained was of higher quality.

Molecular characterization of isolated DNA by RAPD technique was done using 3 arbitrary primers – D1. The PCR products were run on 2% Agarose gel and viewed in Gel Doc Alpha Imager HP. Based on the bands, Dendrogram analysis for frequency similarity was done by using Alpha Imager Software and similarity matrix was generated (Tables 1, 2 & 3). From the matrix, most similarity was observed between sample 4 and 3 for primer D18, sample 4 and 2 for primer D19 and sample 2 and 3 for primer D 20.

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