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Studies on Bacteriocin Producing Abilities of Indigenously Isolated Lactic Acid Bacteria Strains

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Abstract

Lactic Acid Bacteria (LAB) known to produce bacteriocins; a small, ribosomally synthesized antimicrobial peptides considered as next generation antibiotics due to the broad spectrum antibiotic nature. Scanty research was reported on the isolation and screening of different LAB species with high bacteriocin yield. With this lacuna the present study aimed to screen various isolated LAB cultures for their bacteriocin yield abilities and subsequently indentifies the elite LAB species representing higher bacteriocin yield. Initially isolated and preserved LAB cultures were sub-cultured and further screened for their bacteriocin yield abilities on CM media as a production media under submerged state fermentation (SmF) conditions. Based on the bacteriocin yield an elite species was identified, and studied morphologically by growing on different media namely Nutrient agar (NA) media, Acetate agarmedia and MRSmedia. *Pediococcus pentosaceus* strain 2269 obtained from culture collection centre NCIM, Pune, India was used as a standard culture. Microscopic study was performed to screen the visible similarities between the isolate and the standard strain. Further biochemical characterization i.e. Indole, Methyl red, Vogues Proskaeur, Catalase and Carbohydrate fermentation; studies were carried to confirm the culture at Genus level. Finally molecular characterization was done using 16s RNA sequencing followed by phylogenetic analysis (MEGA V-6)to confirm the isolate at species level. Results reported that the culture isolated from the Sanna batter (Sanna 14) was identified as an elite species i.e. *Pediococcus pentosaceus* with higher bacteriocin yield (0.4mg/l) among the three bacteriocin positives cultures reported in the present study. The 16s RNA sequence of the *Pediococcus pentosaceus* culture was submitted to NCBI GenBank with an issued accession number MF183113.

Keywords: Elite species, *Pediococcus pentosaceus*, *Bacteriocins*, Phylogenetic analysis, NCBI-GenBank

Gram positive, non-sporulating, non-motile, non-respiring bacteria which are either rod or coccus shape and posses negative catalase activity are characterized as Lactic acid bacteria (LAB) or Lactobacillales. LAB exhibits similarities in their morphological, metabolic and physiological characteristics and during the fermentation of carbohydrates produce lactic acid as the major end product (Upendra *et al.*, 2016b). LABs are linked with food fermentations, as acidification inhibits the growth of spoilage agents. Their fermentative ability as well as health and nutritional benefits make them industrially important. They tolerate a lower pH range which enables them to outcompete with other bacteria in natural fermentation processes, as they can survive in the increased acidity from organic acid production. *Lactobacillus, Pediococcus, Leuconostoc, Lactococcus, Enterococcus, Melissococcus, Streptococcus, Lactosphaera, Carnobacterium, Weisella* were some of the well known species of LAB (Khandelwal *et al.*, 2015). Metabolites produced by LAB have many applications. Few of the metabolites are antimicrobial

substances such as H_2O_2, CO_2, Acetaldehyde, Diacetyl, D-isomers of amino acids and bacteriocins (Yang *et al.,* 2002). Bacteriocins produced from LAB have received particular attention in recent years due to their potential application in the food industry as natural preservative. These small, ribosomally synthesized antimicrobial proteins or peptides are considered as next generation antibiotics mainly because of their broad spectrum anti microbial nature. Bacteriocins possess inhibitory activity towards closely related bacteria, whereas producer cells are immune to their own bacteriocins (Bhunia *et al.,*1988, Cintas *et al.,*1998, Dalie *et al.,* 2010, Sadeghi *et al.,* 2016). Bacteriocin is believed to be safe for human consumption since it becomes inactive when it comes in contact with digestive enzymes in the stomach. One of the bacteriocins, Nisin has been approved by FDA and is being used as food preservative (Lee *et al.,*2002, Suganthi *et al.,* 2015). Bacteriocins are of interest in medicine as well because they are made by non-pathogenic bacteria that normally colonize the human body such as genus *Lactobacilli* (Wu *et al.,* 2004, Venkatasubramanian and Jeevaratnam, 2013, Maria *et al.,* 2017). Bacteriocins were also suggested for cancer treatment.

The aim of present study is to screen various isolated LAB cultures for their bacteriocin yield abilities and subsequently indentifies the elite LAB species representing higher bacteriocin yield. Initially isolated and preserved LAB cultures were sub-cultured and further screened for their bacteriocin yield abilities on CM media as a production media under submerged state fermentation (SmF) conditions. Based on the bacteriocin yield an elite species was identified, and studied morphologicallyby growing on different media namely Nutrient agar (NA) media, Acetate agarmedia and MRS media. *Pediococcus pentosaceus* strain 2269 obtained from culture collection centre NCIM, Pune, India was used as a standard culture. Microscopic study was performed to screen the visible similarities between the isolate and the standard strain. Further biochemical characterization i.e Indole, Methyl red, Vogues Proskaeur, Catalase and Carbohydrate fermentation; studies were carried to confirm the culture at Genus level. Finally molecular characterization was done using 16s RNA sequencing followed by phylogenetic analysis (MEGA V-6) to confirm the isolate at species level. Results reported that the culture isolated from the Sanna batter (Sanna 14) was identified as an elite species i.e *Pediococcus pentosaceus* with higher bacteriocin yield (0.4mg/l) among the three bacteriocin positives cultures reported in the present study The16s RNA sequence of the *Pediococcus pentosaceus* culture was submitted to NCBI GenBank with an issued accession number MF183113.

MATERIALS AND METHODS

All the chemicals and reagents used during this study were of analytical grade (Merck, India).

Subculturing of preserved LAB isolates: Preserved LAB cultures isolated from selected south Indian fermented food sources were revived initially to MRS liquid broth and further subcultured on solid media such as NA media (Himedia), Acetate agar media (Himedia), and MRS (Himedia) mediaplates, incubated at 37 ^oC for 18-24 Hrs. Fully grown cultures were studied for morphological and microscopic properties (Upendra *et al*., 2013a&b)

Morphological studies on different media: The fully grown colonies of the isolates cultured on different media such as NA media (Himedia), Acetate agar media (Himedia), and MRS were studied morphologically by screening different growth characteristics such as size, shape, color and elevation (Upendra *et al*., 2016b)

Microscopic studies: Subcultures of all the isolates were studied microscopically applying standard Gram's staining procedure. This study was done to determine the purity of the culture. (Upendra *et al*., 2013a)

Biochemical characterization: The biochemical properties of the samples were examined for the strain *Pediococcus pentosaceus* Sanna-14. The Indole, Methyl Red, Vogues Proskaeur tests, Catalase activity, Carbohydrate fermentation and gas production tests were assessed (Nair and Surendran, 2005, Hurtado *et al.,* 2012).

Indole test: Indole test is used to determine the ability of an organism to split amino acid tryptophan to form the compound Indole. Indole production is detected by Kovac's reagent which contains 4(p)-dimethylamino benzaldehyde, this reacts with indole to produce a red coloured compound.1.5% Tryptophanbroth was prepared, sterilized at 121°C for 20 min, inoculated with culture of the test organism and incubated at 37°C for 24 hours. After the incubation period, 0.5 ml of Kovac's reagent was added and observed for the development of red color.

Methyl red test: Methyl Red (MR) test determines whether the microbe performs mixed acids fermentation when supplied glucose. Types and proportion of fermentation products produced by anaerobic fermentation of glucose is one of the key taxonomic characteristics which help to differentiate various genera of enteric bacteria. The standard buffered glucose MR-VP broth (Composition for litre-protease peptone, 7 g; glucose, 5 g; NaCl, 5 g; agar, 10 g) was prepared, sterilized at 121°C for 20 min, 5 ml of prepared broth was inoculated with culture of test organism and incubated at 35°C for 4 days.

Vogues Proskaeur test: Organisms such as members of the *Klebsiella-Enterobacter-Hafnia-Serratia* group produce acetoin as the chief end product of glucose metabolism and form smaller quantities of mixed acids. In the presence of atmospheric oxygen and 40% potassium hydroxide, acetoin is converted to diacetyl, and alpha-naphthol serves as a catalyst to bring out a red complex. The standard buffered glucose MR-VP broth (Composition for litre-protease peptone, 7 g; glucose, 5 g; NaCl, 5 g; agar, 10 g) was prepared, sterilized at 121°C for 20 min. 5 ml of the prepared broth was inoculated with culture of test organism and incubated at 35°C for 24 hrs. At the end of the incubation period to the 1 ml of broth 0.6mL of 5% alpha naphthol followed by 0.2 mL of 40% KOH was added and the tube was shaken gently to expose the medium to atmospheric oxygen and allow the tube to remain undisturbed for 10 to 15 minutes.

Catalase test: Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. To find out if a particular bacterial isolate is able to produce catalase enzyme, small inoculum of bacterial isolate is mixed into hydrogen peroxide solution (3%) and is observed for the rapid elaboration of oxygen bubbles formation. The lack of catalase is evident by a lack of or weak bubble production. 4 to 5 drops of 3% $H_2O_2$was taken in a test tube and using a wooden applicator stick, a small amount of test organism colony from culture plate incubated at 18-24-was picked and placed into the test tube. The tube was placed against a dark background and observe for immediate bubble formation (O_2 + water = bubbles) at the end of the wooden applicator stick

Screening of subcultured LAB isolates for their Bacteriocin yield abilities

SmF production of Bacteriocins: Single colonies of the selected LAB isolates were transferred aseptically into 100 ml of seed culture media (MRS broth), and incubated at 37°C for 18-24 hours. Further the 10 % of the seed culture of each LAB isolates was inoculated in 100 ml of production media (CM media) composed-Sucrose (2.86%), Tryptone (0.5%), Yeast extract (1%), Tween 80 (0.3%), Magnesium sulphate (0.02%), Sodium Chloride (0.81%) K_2HPO_4 (1.91%) Ascorbic acid (0.05%) and Agar (1.2%) taken in four different 250 ml conical flasks. All the inoculated production media was kept at 37°C, 6.5pH, and 150 rpm for 72 Hrs (Biswas *et al.*, 1991, Aasen *et al.*, 2000, Upendra *et al.*, 2016**).**

Downstream processing of Bacteriocins: After 72 hours of incubation, the bacteriocin produced in the fermentation broth was separated by centrifugation (10000 rpm) for 21 minutes at 4°C. The supernatant obtained, is transferred to a 250 ml Erlenmeyer flask and pellet is washed off. The pH of the fermentation broth was adjusted to 7.0 with 3M NaOH and was filtered using a cellulose acetate filter syringe with 0.22µm pore size (Millipore, USA). The filtrate consists bacteriocin was added with phosphate buffer in order to avoid antagonism by hydrogen Peroxide. Crude extract was treated with solid ammonium sulphate 40, 50, and 60% saturation. The mixtures were stirred for 2 h at 4°C and later centrifuged at

14,000 rpm for 1 h at 4°C. The pellet was resuspended in 25 ml of 0.05 M potassium phosphate buffer having pH 7.0. Dialysis was carried out against the same buffer for 12 h in spectrapor dialysis tubing. Assay of the bacteriocin activity was carried out and titer was determined (Upendra *et al.,* 2016b).

Quantitative determination of purified Bacteriocins: 10 ml of purified Bacteriocin samples obtained from the batch cultures of all the three LAB isolates was qualitatively studied by measuring the absorbance spectra in between 200-240nm in UV Visible, spectrophotometer with respect to standard Bacteriocin (Nisin) obtained for Anand Agriculture University, Anand, Gujarat, India. The absorbance maxima of the purified samples were compared with the absorbance maxima of authenticated standard bacteriocin (Nisin) sample (Pavels *et al.,* 2008, Upendra *et al.,* 2016b).

Molecular characterization of bacteriocin high yield LAB isolate: Total genomic DNA from the elite LAB species was isolated by N- Cetyl- N, N, N-trimethyl- ammonium bromide (CTAB) method. The quantity of the isolated DNA was screened in UV-VIS spectrophotometer (Vivaspec Biophotometer, Germany). From the stock 1µl DNA was mixed with 49-µl sterile distilled water to get 50 times dilution. The A260/A280 ratio was recorded to check the purity of DNA preparation. PCR amplification of 16s region was done in 20 µl of reaction mixture containing PCR buffer, 1X (Kappa, SA); $MgCl_2$, 3 mM; dNTP mix, 0.25 mM; *Taq* DNA polymerase, 0.05 U; primer, 1 picomol and template DNA, 50 ng, using 27F, 1492R primer set and sterile nuclease free water as negative control (Sambrook and Russell, 2001). The amplified DNA was sequenced and was analyzed for homology using BLASTN. Phylogenetic analysis was performed by using the MEGA V6 software (Tamura *et al.,* 2011). Sequences were aligned and a rooted neighbor-joining tree was constructed by using *Pediococcus pentosaceus* as an out group species. The 16s rRNA gene sequence of *Pediococcus pentosaceus* strain was registered in the GenBank database NCBI-USA with an accession number (Nettles *et al.,* 1993).

RESULTS AND DISCUSSION

Subculturing of preserved LAB isolates: Subcultured LAB isolates on MRS media were shown in Fig. 1. Under aerobic conditions, colonies were found to be small size of 0.5 mm in diameter, round shaped, gray coloured and slightly convex with smooth edges. Under anaerobic conditions the colonies were small and appeared as round ovals with smooth edges.

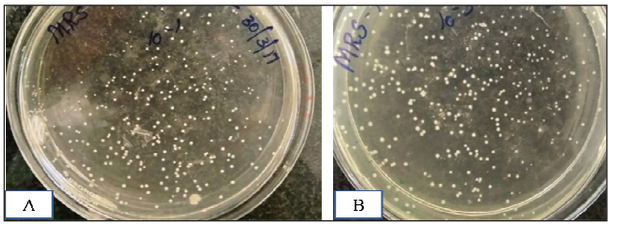
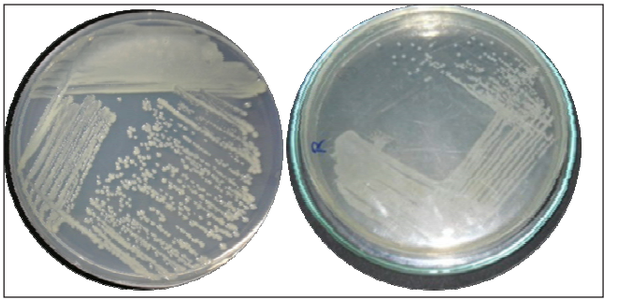


Fig. 1: MRS plates showing subcultured LAB isolates incubated at 37°C for 24 hours: A. Lab cultures grown under aerobic conditions, B. Lab cultures grown under anaerobic conditions



Pediococcus pentosaceus 2296 (NCIM, Pune) Sanna-14 isolate

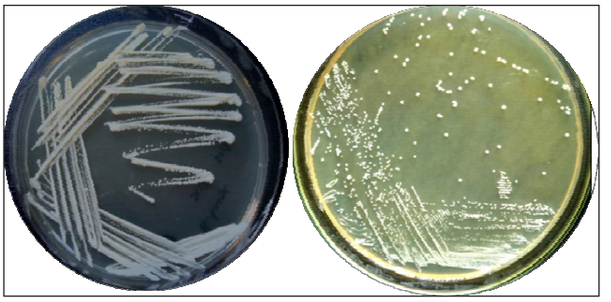
Fig. 2: NA plates showing standard *Pediococcus pentosaceus* 2296 (NCIM, Pune) culture and subcultured Lab species (Sanna 14) incubated at 37°C for 24 hours

Morphological studies on different media: All the subcultured LAB isolates were studied morphologically on and compared with the standard culture *Pediococcus pentosaceus* 2296 (NCIM), by screening different growth characteristics such as size, shape, color and elevation. Fully grown subcultured LAB isolates and the standard *Pediococcus pentosaceus* 2296 culture obtained from NCIM Pune, on different media was represented in Fig. 2 Nutrient Agar (NA) media, Fig. 3 Rogosa and Sharpe (MRS) media and

Fig.4 Acetate agar (AA) media respectively. The culture was observed to grow in the selective media acetate agar. The colonies were found to be grayish white in color, round in shape, concave in nature and have smooth edges (Table 1).

Table 1: Morphology comparison of standard *Pediococcus pentosaceus* 2296 (NCIM, Pune) and the subcultured lab isolate (Sanna 14)

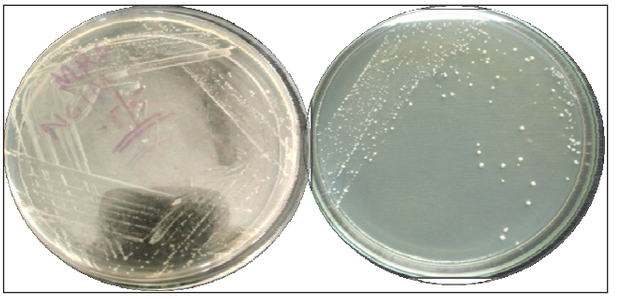
Type of media	*Pediococcus pentosaceus* 2296 (NCIM, Pune)	Sanna-14, isolate
1. Nutrient agar		
Shape:	Round, convex	Round, convex
Color:	Grayish white	Grayish white
Edges	Smooth	Smooth
2. MRS agar		
Shape:	Round, convex	Round, convex
Color:	Gray	Gray
Edges:	Smooth	Smooth
3. Acetate agar		
Shape:	Round, concave	Round, concave
Color:	Gray	Gray
Edges:	Smooth	Smooth



Pediococcus pentosaceus 2296 (NCIM, Pune) Sanna-14 isolate

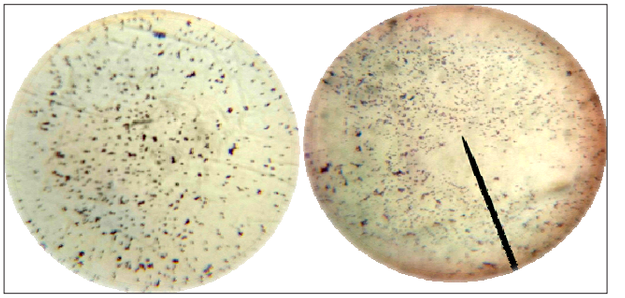
Fig. 3: MRS plates showing standard *Pediococcus pentosaceus* 2296 (NCIM, Pune) culture and subcultured Lab species (Sanna 14) incubated at 37°C for 24 hours

Microscopic screening: Microscopic properties of all the subcultured LAB isolates were studied and compared with the standard *Pediococcus pentosaceus* 2296 (NCIM, Pune) culture microscopically applying standard Gram's staining procedure. Studies were performed to determine the purity of the culture and were found to be purple in color indicating gram positive cocci occurred as tetrads.



Pediococcus pentosaceus 2296 (NCIM, Pune) Sanna-14 isolate

Fig. 4: AA plates showing standard *Pediococcus pentosaceus* 2296 (NCIM, Pune) culture and subcultured Lab species (Sanna 14) incubated at 37°C for 24 hours



Pediococcus pentosaceus 2296 (NCIM, Pune) Sanna-14 isolate

Fig. 5: Gram's stain of standard *Pediococcus pentosaceus* 2296 (NCIM, Pune) culture and subcultured Lab species (Sanna 14) (100X) showing purple colored Gram-positive cocci

Biochemical characterization studies: The biochemical properties of the samples were examined for the strain *Pediococcus pentosaceus* Sanna-14, using Bergey's Manual (Whitman *et al.*, 2012). The Indole, Methyl Red, Vogues Proskaeur tests, Catalase activity, Carbohydrate fermentation tests were assessed and the results were shown in the Fig. 6 and Table 2.

Screening of subcultured LAB isolates for their Bacteriocin yield abilities: Subcultured LAB isolates were grown under submerged fermentation (SmF)

conditions to screen their bacteriocin yield abilities. At the end of 72 Hrs of fermentation the acidified broth was taken for the purification of bacteriocines. Crude extract of Bacteriocins was purified further through Ammonium sulphate precipitation method and the purified bacteriocins was quantified UV Spectrophotometrically (Fig. 7). Among the 10 cultures screened three Lab cultures were found positive for bacteriocin production and the Sanna 14 culture isolated from the Sanna batter has shown the higher bacteriocin yield (0.4 mg/l) among all the bacteriocin positive cultures.

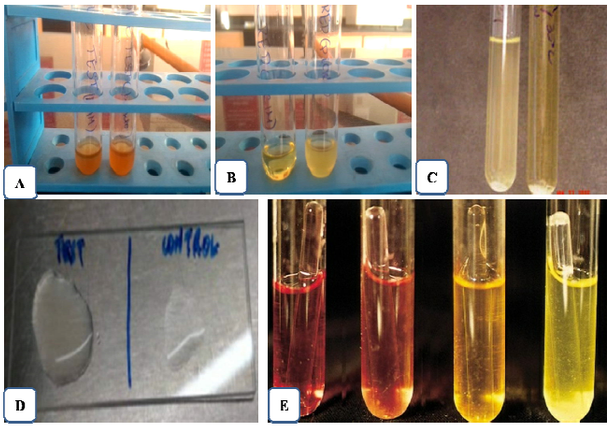


Fig. 6: Comparison biochemical tests of standard *Pediococcus pentosaceus* 2296 (NCIM, Pune) culture and subcultured Lab species (Sanna 14) A. Indole test, B. Methyl red test, C. Vogues Proskaeur test, D) Catalase test, E) Carbohydrate fermentation

Table 2: Results of the Biochemical characterization studies

Sl. No.	Test	Colour	Results
1	Indole test		Negative
2	Methyl red		Negative
3	Vogues Proskaeur		Negative
4	Catalase test		Negative
5	Carbohydrate fermentation		Positive

Molecular characterization of bacteriocin high yield LAB isolate: The identified elite species (Sanna 14 isolate), was characterized at molecular level by 16s rRNA sequencing. Fig. 8 represents the PCR amplified fragment of the isolated DNA. The16s RNA sequence information was aligned and compared with all the 16s rRNA gene information available in the GenBank NCBI using Multiple Sequence Alignment BLAST comparison tool (Fig. 9).

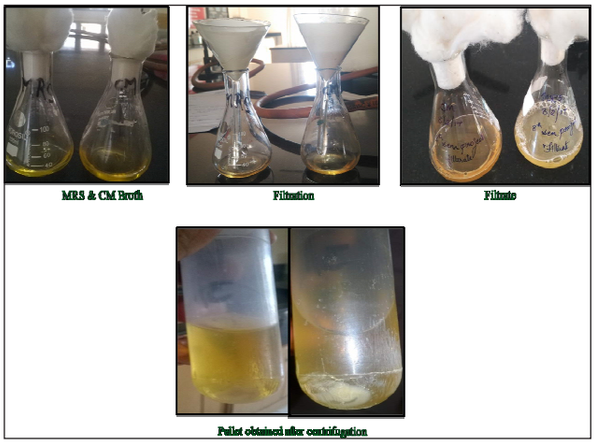


Fig. 7: SmF process of bacteriocin production and DSP of bacteriocin from the fermented broth

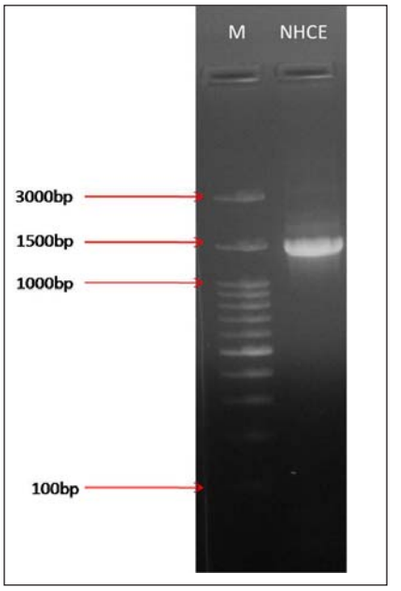


Fig. 8: The gel electrophoretic results of the PCR amplified product of Sanna 14 isolate

Blast was designed to identify local regions of sequence similarity which report multiple discrete regions of sequence similarity between a query sequence and a subject sequence in a database. Color of the boxes corresponds to the score (S) of

the alignment, with red representing the highest alignment scores. The higher the alignment score, the more significant the hit.

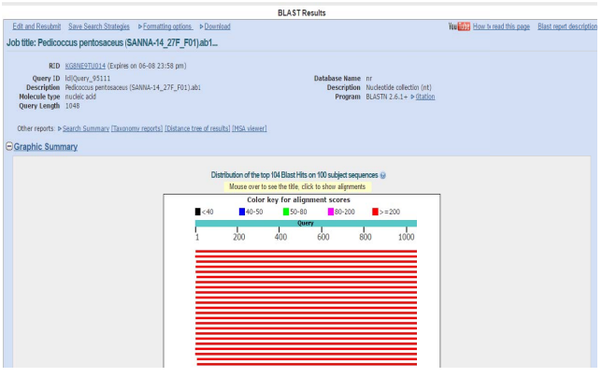


Fig. 9: BLAST results of Sanna 14 isolate

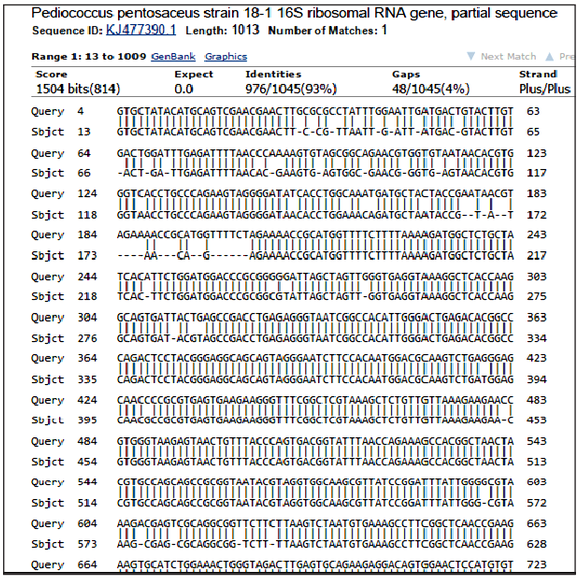


Fig. 10: The multiple sequence alignment of Sanna 14 isolate with maximum aligned *Pediococcus pentosaceus* 18-1 16 s r RNA gene sequence

All the sequences in the reference sequence database show significant sequence homology to the Sanna 14 sequence. The multiple sequence alignment shown that the Query sequence was aligned maximum with the highest alignment score 1504, of a *Pediococcus pentosaceus* 18.1 16 sRNA gene sequence with accession number – KJ477390.1 (Fig. 10). The expect value found to (0.0) and 93% similarities was seen for the highest aligned sequence. Sequence annotation was submitted to NCBI GenBank with an issued accession number MF183113.

Table 3: Alignment statistics for multiple sequence alignment of Sanna 14 isolates sequence and *Pediococcus pentosaceus* strain 18-1 16S ribosomal RNA gene, partial sequence ID: KJ477390. 1 Length: 1013, Number of Matches: 1, Range 1: 13 to 1009.

Alignment statistics for match #1				
Score	**Expect**	**Identities**	**Gaps**	**Strand**
1504 bits (814)	0.0	976/1045 (93%)	48/1045 (4%)	Plus/ Plus

The phylogenetic analysis of the 16s rRNA gene sequence was aligned using Clustal W in MEGA V6 software. Phylogenetic tree was constructed using Neighbor joining and bootstrap method and inference of the tree was given through maximum likelihood and maximum parsimony method. Phylogenetic analysis showed its close relation with different strains of the *Pediococcus pentosaceus* species (Fig. 11 A & B).

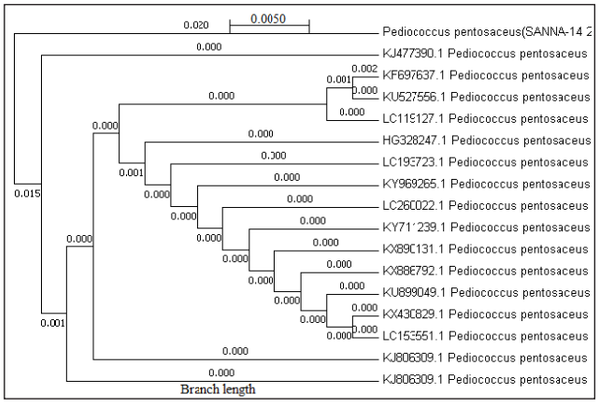


Fig. 11: Phylogenetic tree based upon the neighbor-joining method of partial 16S rDNA sequences of Sanna 14 isolate. **A.** Rectangular Phylogenetic inference of Sanna 14 isolates, **B.** Circular Phylogenetic inference of Sanna 14 isolates

CONCLUSION

In the present study, initially LAB isolated from south Indian food such as Sanna were revived and characterized using standard microbiological techniques such as morphological, microscopic

and biochemical characterization methods. Further SmF process was carried out for the production of Bacteriocin. Purified Bacteriocins from the fermented broth was quantified using UV spectrophotometry with the authenticated standard bacteriocin (Nisin). Based on the bacteriocin yield the elite species Sanna 14 isolates (0.4 mg/l bacteriocin) was selected and characterized molecularly by performing 16s rRNA sequencing methodology. Phylogenetic analysis of the Sanna 14 sequence by MEGA V 6 reveals that the studied organism was found to be *Pediococcus pentosaceus* with highest bacteriocin yield 0.4 mg/l. In conclusion in the present study attempt were successful in producing bacteriocin for indigenous cultures of LAB isolated from different south Indian fermented food samples. The 16s RNA sequence of the *Pediococcus pentosaceus* culture (Sanna 14) was submitted to NCBI GenBank with an issued accession number MF183113.

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