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Isolation and Characterization of *Pediococcus pentosaceus* from *Idly* Batter: A Traditional South Indian Fermented Food Source

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Lactic acid bacteria (LAB) are protective and generally recognized as safe (GRAS) organisms that are involved in fermentation process. *Idly* batter is a traditional fermented food source in south India and distribution of LAB varies with source of batter preparation and seasons. Thus, an attempt was made to isolate LAB from *idly* batter. A total of 40 cocci were obtained out of which 18 exhibit wide spectrum of antimicrobial activity against Gram positive and negative organisms. The antimicrobial activity was lost completely after treatment with protease suggesting bacteriocinogenic in nature. These potent bacteriocinogenic isolates were clustered into four based on the RAPD and further grouped into six based on physiological, and biochemical characterization. One isolate from each group were further characterized using various molecular tools. The 16S rRNA gene sequencing of homofermentative isolates exhibited 98–100% homology with *Pediococcus pentosaceus*. These isolates obtained from *idly* batter may have a significant role imposing health benefits.

Key words: *Idly* batter, *Pediococcus*, *Leuconostoc*, 16S rRNA.

Lactic acid bacteria (LAB) are important group of fermentative bacteria used in several household and industries as starter cultures to ferment food products. They contribute to the improvement of the taste, as well as to preservation and microbial safety to the food[1] by producing various antibacterial and antifungal substances[2]. Hence, they prevent outgrowth of spoilage pathogenic bacteria in conservation of foods. In the present study, LAB is been isolated from *Idly* batter a legume-based fermented food in India. The essential microbes responsible for fermentation are found to be naturally present in the ingredients[3]. Thus, the objective of this study is to isolate and identify LAB from *idly* batter exhibiting wide spectrum of antimicrobial activity.

MATERIALS AND METHODS

Isolation and screening for antimicrob--ial activity: LAB was isolated from *idly* batter, after serial dilutions with 0.85% saline, plated on MRS agar and incubated anaerobically. White clear elevated colonies were selected and pure cultures were stored in MRS broth with 30% (v/v) glycerol at -20ºC. Indicator organisms used in this study

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were procured from the Microbial Type Culture Collection (MTCC) from the Institute of Microbial Technology, Chandigarh (Table 1), maintained and propagated in MRS and Soyabean Casein digest broth. The isolates were grown in MRS broth and antimicrobial activity was evaluated by agar well diffusion assay as described earlier[4]. Isolates inhibiting various LAB, Gram positive and Gram negative pathogenic indicator organisms (Table 1) were considered as potent isolates and selected for further characterization studies. The antimicrobial substance was treated with 3mg/ml of protease in 10mM citrate buffer pH 3.0 (Sigma, India) and the activity was evaluated against *Staphylococcus aureus* [5]. The control was also processed in similar way without enzyme.

Physiological and biochemical characterization: The physiological and biochemical characterization of the isolates were performed employing methods as described earlier[4]. The homo–hetero fermentation was carried using the differential broth as described earlier [6]. Acid production from various carbohydrates was determined using HiCarbo Kit (Himedia, Mumbai) as per the manufacturers recommended procedure.

Genomic DNA isolation: The genomic DNA was isolated for the potent isolates using 2 ml overnight cultures in MRS broth[7]. The purity of DNA was checked using 0.8% agarose gel electrophoresis run in TBE buffer (45 mmol $l^{\times 1}$ Tris -45 µmol $l^{\times 1}$ Borate-1 mmol $l^{\times 1}$ EDTA), stained with ethidium bromide and further quantified using spectrophotometer (Shimadzu, Japan).

RAPD-PCR analysis: The primers used for the RAPD reactions were M13 (5' - GAGGGTGGCGGTTCT-3') and R1 (5'-TCAGCCCCTA-3'). Using the genomic DNA, PCR was performed with 50 µl reaction mixture containing 1X buffer, 1.5 mmol l^{-1} $MgCl_2$, 200 µmol l^{-1} of dNTP mix, 0.5 µmol l^{-1} of primer (Eurofins Genomics, India Pvt. Ltd., India), 50 ng of genomic DNA, and 1U of Taq DNA polymerase (Merck Biosciences, India). The DNA was amplified with Mastercycler Gradient (Eppendorf, Germany) with initial denaturation at 94ºC for 5 min, 30 cycles of 94ºC for 40 s, annealing at 40ºC (M13) and 29ºC (R1) for 1 min followed by ramping to 72ºC for 0.5ºC/s and extension for 2 min with final extension at 72ºC for 5 min. The products were checked using 1.5% agarose gel electrophoresis with TBE buffer and stained with ethidium bromide.

16S rRNA gene amplification and sequencing: The 16S rRNA gene was amplified in 50 µl reaction mixture containing 1X buffer, 1.5 mmol l^{-1} $MgCl_2$, 200 µmol l^{-1} of dNTP mix, 50 ng of template DNA, 1 U of Taq DNA polymerase and 50 µmol l^{-1}

Table 1. Antibacterial spectrum of 6 isolates selected from RAPD.

Indicator Bacteria	Isolates					
	C1		C2	C3		C4
	VJ35	VJ31	VJ13	VJ41	VJ56	VJ49
Lactobacillus plantarum MTCC 6161	10±1	10±3	11±1	10±4	10±1	10±1
Lactobacillus fermentum MTCC 1745	11±1	11±1	11±1	10±1	11±2	11±1
Lactobacillus brevis MTCC *1750*	11±1	10±2	11±1	11±1	10±1	11±1
Leuconostoc mesenteroids MTCC 107	12±2	11±1	12±2	11±1	11±2	12±2
Lactococcus lactic MTCC 3038	18±2	16±2	18±2	17±2	17±1	16±2
Lactococcus lactis MTCC 440	13±1	11±2	12±2	11±2	11±1	11±1
Micrococcus luteus MTCC 106	19±2	18±1	20±2	18±1	18±1	17±2
Listeria monocytogenes MTCC 657	18±2	18±2	21±3	19±1	19±1	18±2
Bacillus subtilis MTCC 619	20±1	16±2	18±2	16±1	19±3	20±2
Aeromonas hydrophila MTCC 1739	22±2	20±2	21±2	20±2	21±3	20±2
Pseudomonas aeruginosa MTCC 2295	19±2	18±1	20±3	17±2	20±2	19±2
Escherichia coli MTCC 728	14±1	14±5	15±5	14±1	11±1	11±2

Inhibition zone in mm inclusive of well diameter 6mm. Values are means of 3 independent experiments performed in duplicate while the range is given in parentheses

Table 2. Physiological and biochemical characteristics of the clustered isolates.

	C1		C2	C3		C4
Isolate Number	VJ31	VJ35	VJ13	VJ41	VJ56	VJ49
Acetoin production	+	+	+	+	-	+
Arginine hydrolysis	+	+	+	+	-	+
Growth at pH 9.5	+	-	+	+	-	-
Salt Tolerance (6.5%)	+	+	+	±	+	-
Carbohydrate fermentation†						
Maltose	+	+	-	+	-	-
Galactose	+	±	-	-	-	-
Rafinose	+	+	-	-	-	+
Trehalose	+	-	+	+	-	-
Melibiose	+	+	-	-	-	+
Sucrose	-	-	-	±	±	-
Mannose	+	+	+	+	+	+
Inulin	+	-	-	±	+	+
Salicin	+	+	-	-	-	-
Cellobiose	+	+	-	±	-	-

(+) good growth; (±) weak growth;(–) no growth;

of primers forward fKJ (5'-CATTGGGACT GAG A CACTGC-3') and reverse rKJ (52 -CACC GC GA CATGCTGATTC-3') whose amplicon size is approximately 1kb covering V3 to V8. The amplification has an initial denaturation at 94ºC for 5 min followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 59.5ºC for 1 min and extension at 72ºC for 2 min, and a final extension at 72ºC for 5 min using Mastercycler Gradient (Eppendorf, Germany). Amplified product was checked by electrophoresis using 1% agarose in TBE buffer, was cleaned using PCR clean-up kit (Merck Bioscience, India) and sequenced using the automated DNA sequencer (Macrogen Inc., Seoul, Korea). The reference sequence was collected from GenBank using BLASTn and aligned using ClustalW. The phylogenetic tree was constructed using Neighbour-Joining method with MEGA 5.0 software after resampling 1000 times with bootstrap analysis.

Nucleotide accession numbers: The nucleotide sequences of the isolates were deposited in GenBank and the accession numbers are JN573609 to JN673614.

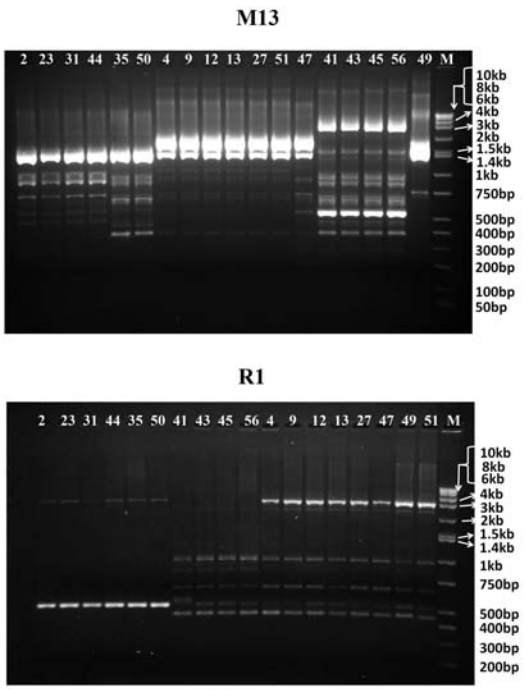

Fig. 1. RAPD-PCR of the 19 potent antimicrobial substance producing isolates. Lane 20 is wide range DNA marker (D7058, Sigma) and lane 1 to lane 19 is the isolates in group.

RESULTS AND DISCUSSION

Idly an easily digestible food, prepared from fermented batter and steam cooked is the most preferred food in south India. The predominant isolate in this study was *Pediococcus* and *Leuconostoc* which could have been possibly obtained from dehulled black gram whereas in earlier reports *Lactococcus lactis* has been isolated from *idly* batter[8]. This variation may be due to source of batter preparation and season that affect the prevalence of bacteria and yeast in the batter[9].

Forty elevated colonies which were Gram positive, catalase negative, acid producing, cocci were isolated from *idly* batter. They were grouped into 36 homofermentative and 4 heterofermentative, which were screened for antimicrobial property against various LAB and pathogenic organisms. The heterofermentative isolates were not potent hence were not chosen for further studies. A total of 18 homofermentative isolates showed good antimicrobial activity which was lost after treatment with protease thus confirmed bacteriocinogenic property[5]. These isolates were further clustered using RAPD, physiological and biochemical properties. The 18 isolates were clustered into 4 based on the RAPD (Fig. 1.) and those differing with physiological and biochemical properties (Table 2) within each cluster were selected for further analysis. The 16S rRNA gene product sequence of homofermentative isolates revealed 98–100% homology with *P. pentosaceus* (Fig. 2.) using BLASTn of NCBI and the phylogenetic tree constructed based on Neighbour-Joining method revealed all to be *P. pentosaceus*. The V3 and V6 region of 16S rRNA gene effectively distinguish between closely related organisms[10] so this region was selected for amplification. All isolates produce acid from dextrose, fructose, mannose and esculin while none of the isolates produces acid from lactose, L & D-arabinose, rhamnose, xylose, ribose, sorbose, xylitol, mannitol, inositol, dulcitol, sorbitol, melezitose, adonitol, malonate, glycerol, citrate, glucosamine and gluconate. Since they all grow at 15, 45 and 37°C while none grow at 50°C, they are confirmed as *P. pentosaceus* differing at strain level because of variation in their biochemical and physiological characteristics (Table 2).

In conclusion, the predominant bacteriocinogenic cocci from *idly* batter are *Pediococcus pentosaceus* exhibiting wide spectrum of activity against several pathogenic Gram positive and Gram negative organisms. Thus, the potent bacteriocinogenic isolates have to further be evaluated for its probiotic potential which may have a beneficial role in health.

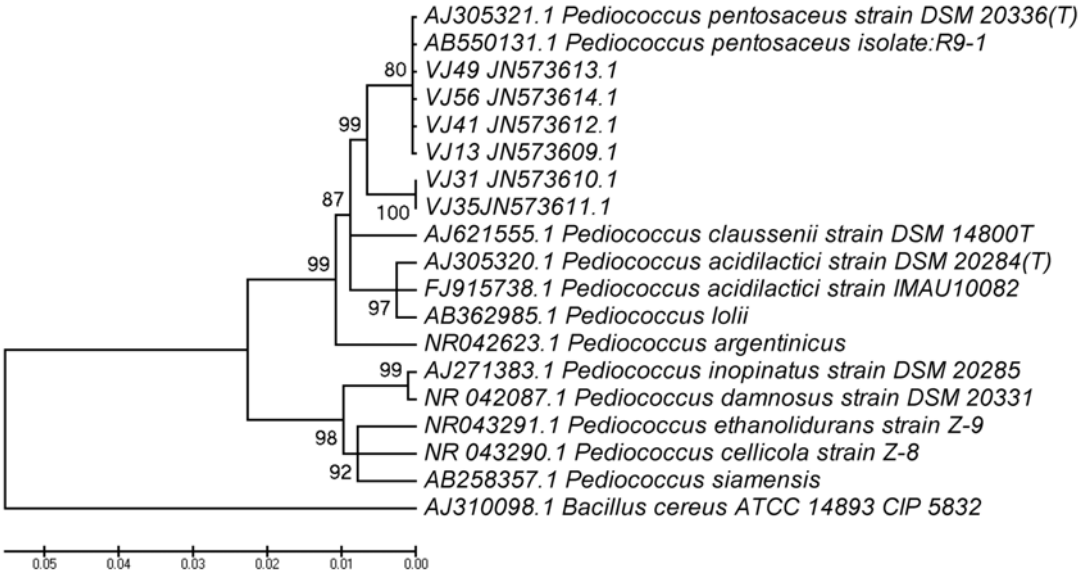

Fig. 2. The phylogenetic tree constructed by Neighbour-Joining method.

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