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Molecular Characterization of Bacteriocinogenic *Lactobacillus* Species Isolated from Fermented *Uttapam* Batter

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Lactic acid bacteria (LAB) are protective and generally recognized as safe (GRAS) organisms. The antimicrobial compounds produced by LAB act effectively against several pathogenic organisms, hence they are well-employed as food preservative. This study aims at isolation and characterization of potent bacteriocinogenic lactobacilli from fermented *Uttapam* batter. Eighteen bacilli showing wide spectrum of inhibitory property against Gram positive and negative organisms were grouped using RAPD, physiological and biochemical characteristics. Eight bacteriocinogenic potent isolates having variation were further identified using 16S rRNA gene sequencing and belonged to *Lactobacillus plantarum* with 99% sequence homology. Using multiplex PCR the isolates were identified using various molecular tools as *Lactobacillus pentosus, L. plantarum* ssp. *argentoratensis*, *L. plantarum* and *L. plantarum* ssp. *plantarum*. These isolates have to be evaluated further for several applications, that turns out to be beneficial for humans.

Key words: *Uttapam* batter, *Lactobacillus*, Bacteriocinogenic, RAPD, 16S rRNA, Multiplex PCR.

Lactic acid bacteria (LAB) are non-pathogenic Gram-positive organisms fermenting several food products contributing to the flavour, taste, texture having beneficial influence on nutritional and sensory characteristics[1]. They have not only been used for food preservation but also as natural probiotics[2]. These organisms produce wide assortment of antimicrobial compounds such as organic acids, low-molecular weight compounds and antimicrobial peptides referred as bacteriocins. Currently, enormous interest has been put forth to probe the potential use of bacteriocins because of their effectiveness against various bacteria and certain fungal strains[3]. Isolation and characterization of bacteriocinogenic LAB from traditional Indian food sources like appam and idly batter has previously been reported[4, 5]. But this was the first attempt to isolate and characterize bacteriocinogenic lactobacilli from fermented *Uttapam* batter.

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MATERIAL AND METHODS

Isolation of LAB from Fermented *Uttapam* Batter

LAB was isolated from fermented *Uttapam* batter, used for preparation of south Indian pancakes. The batter is made by soaking

Table 1. Antibacterial profile of 8 most potent isolates against indicator organisms.

	C1	**C2**			**C3**		**C4**	**C5**
	SJ65	SJ5	SJ22	SJ6	SJ37	SJ40	SJ33	SJ9
Lactococcus lactis MTCC 3038	18±2	17±2	18±3	17±2	15±2	17±3	18±3	17±3
Lactobacillus fermentum MTCC 1745	12±1	12±2	12±2	11±1	10±1	11±1	13±2	10±1
Brevibacterium casei MTCC 1530	10±1	11±1	11±2	10±1	10±1	11±2	11±1	10±1
Leuconostoc mesenteroides MTCC 107	11±1	12±1	11±1	11±1	11±2	11±2	11±1	11±1
Lactobacillus brevis MTCC 1750	11±1	10±1	11±1	11±1	10±1	10±2	11±2	12±2
Staphylocccus aureus MTCC 737	18±3	17±2	17±3	16±2	16±4	16±3	15±3	18±3
Micrococcus luteus MTCC 106	18±2	17±2	16±3	16±2	14±3	16±3	16±2	18±4
Bacillus subtilis MTCC 619	18±2	17±3	17±2	18±3	11±2	16±2	16±2	18±3
Vibrio parahemolyticus MTCC 451	16±3	16±3	15±2	16±2	14±4	15±2	16±2	17±3
Pseudomonas aeruginosa MTCC 2295	18±2	17±3	18±2	16±3	14±2	15±2	19±4	16±3

Inhibition zone expressed in millimeters inclusive of well diameter 6mm. Values are means of three independent experiments performed in duplicates with standard deviation.

Table 2. Biochemical and physiological characterization for the clustered eight isolates.

	C1	C2			C3		C4	C5
	SJ65	SJ5	SJ22	SJ6	SJ37	SJ40	SJ33	SJ9
Growth at temperature (15ÚC)	+	+	±	+	±	+	+	+
Growth at pH (3.5)	+	+	+	–	–	–	–	+
Acetoin production	+	+	+	–	–	+	–	+
Carbohydrate fermentation								
Galactose	–	+	+	–	+	–	+	–
Trehalose	+	–	–	–	+	+	–	–
Sucrose	–	–	+	±	+	+	+	+
Inulin	+	–	+	+	+	+	+	+
Salicin	+	+	+	–	+	±	–	+
Ribose	+	–	+	–	+	–	–	+
Cellobiose	+	–	+	+	+	+	±	+
Melezitose	+	–	–	–	–	–	–	–

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

rice and urad dhal (black lentil) in the ratio of 3:1 with few fenugreek seeds for 4–6 h, ground, mixed with salt and allowed to ferment for 60 h. Serial dilutions of this batter was done, plated on MRS (de Mann Rogosa Sharpe) agar, and incubated anaerobically at 37ºC. Creamy, opaque, elevated colonies were randomly picked and pure cultures were obtained and preserved in MRS broth with 30% glycerol stocks at -20ºC. Gram staining was done and isolates was selected for further study.

Screening of lactobacilli for its bacteriocinogenic property

Colonies were grown in MRS broth and the cell free supernatant was concentrated to tenfold using Rota evaporator (Buchi, Switzerland) and evaluated for antimicrobial activity as described earlier[4] against various Gram positive and negative pathogenic indicator organisms (Table 1) procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, India and maintained in soya-bean casein digest broth/agar. Bacteriocinogenic property was evaluated using cell free supernatant concentrate treated with 3mg/ml final concentration

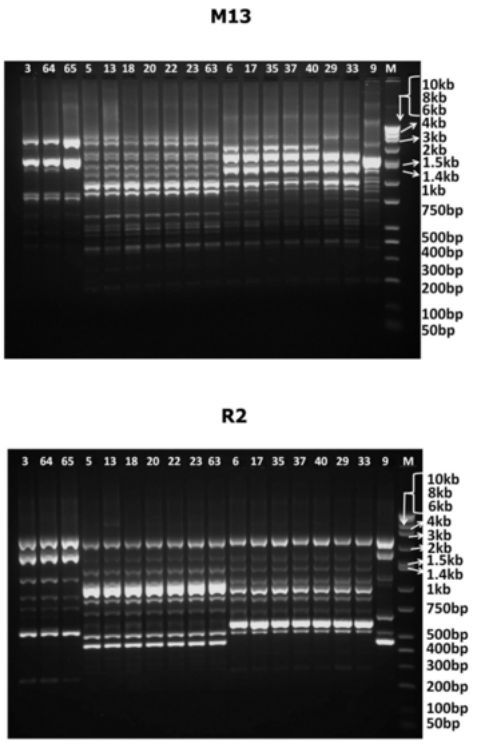

Fig. 1 RAPD-PCR of the 18 bacteriocinogenic isolates for RAPD M13 and RAPD2 primers. Lane M is wide range DNA marker (D7058, Sigma) and lane 1 to lane 18 is the isolates run in clusters. Cluster 1 – SJ3, 64, 65; Cluster 2 – SJ5, SJ13, SJ18, SJ20, SJ22, SJ23, SJ63; Cluster 3 – SJ6, SJ17, SJ35, SJ37, SJ40; Cluster 4 – SJ29, SJ33 and Cluster 5 – SJ9

of acid protease in 10mM citrate buffer pH 3.0 and incubated at 37°C. A control was also processed in a similar manner without enzyme. The inhibition was checked against *Staphylococcus aureus* [6].

Phenotypic characterization studies for *Lactobacillus* isolates

Biochemical and physiological properties of the isolates was done as described earlier[4]. The lactic acid configuration was determined enzymatically using D- and L-lactate dehydrogenases (Sisco Research Laboratories, India). Ability to ferment various sugars was examined using Hi-Carbohydrate kit, (Hi-Media, India).

Genomic DNA Extraction and RAPD analysis of 18 isolates

The genomic DNA was isolated from 2ml of overnight culture of MRS broth [7] and purity was determined spectrophotometrically (Shimadzu, Japan). Genomic DNA was used for RAPD analysis, using M13 (5'-AGGGTGGCGGTTCT-3') and R2 (5'-GGCGACCACTAG-3') primers (Eurofins Genomics, India Pvt Ltd). PCR reaction mixture of 50 µl containing 1x buffer, 1.5 mM of $MgCl_2$, 200 µM of dNTP, 0.5 µM of primer, 50 ng of genomic DNA, and 1U of Taq DNA polymerase (Merck Bioscience, India). DNA was amplified using Master Cycler Gradient (Eppendorf, Germany) with conditions consisting of initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 40°C (M13) and 38°C (R2) for 1 min followed by ramping to 72°C at 0.5°C/s, extension at 72°C for 2 min and final extension at 72°C for 5 min. The amplified products were resolved by 1.5% agarose gel electrophoresis using TBE (Tris Borate EDTA) buffer at 100 V and stained with ethidium bromide.

16S rRNA analysis

The 16S rRNA gene was amplified for the selected isolates using primers, fKJ (5'-CATTGGGACTGAGACACTGC-3') and rKJ (5'-CACCGCGACATGCTGATTC-3') (Eurofins Genomics India Pvt Ltd) that amplifies V3 to V8 whose product size is around 1kb. The DNA was amplified in 50 µl reaction mixtures containing 1x buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 0.5 µM of primer, and 1U of Taq DNA polymerase (Merck Bioscience, India). Amplification included initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 59.5°C for 1 min, extension at 72°C for 2 min, and a final extension at 72°C for 5 min. The PCR products were checked using 1% agarose gel electrophoresis. The PCR product was cleaned using clean-up kit (Merck Bioscience, India) and sequenced at Macrogen Inc., Korea. BLASTn was done and reference sequences were collected from NCBI and aligned using ClustalW. The pairwise evolutionary distance was done by Kimura-2-parameter and phylogenetic tree was constructed using Neighbour-Joining method with MEGA 5.0 software after resampling 1000 times with bootstrap analysis. The 16S rRNA gene sequences were deposited in GenBank (accession numbers JN573616 to JN573623).

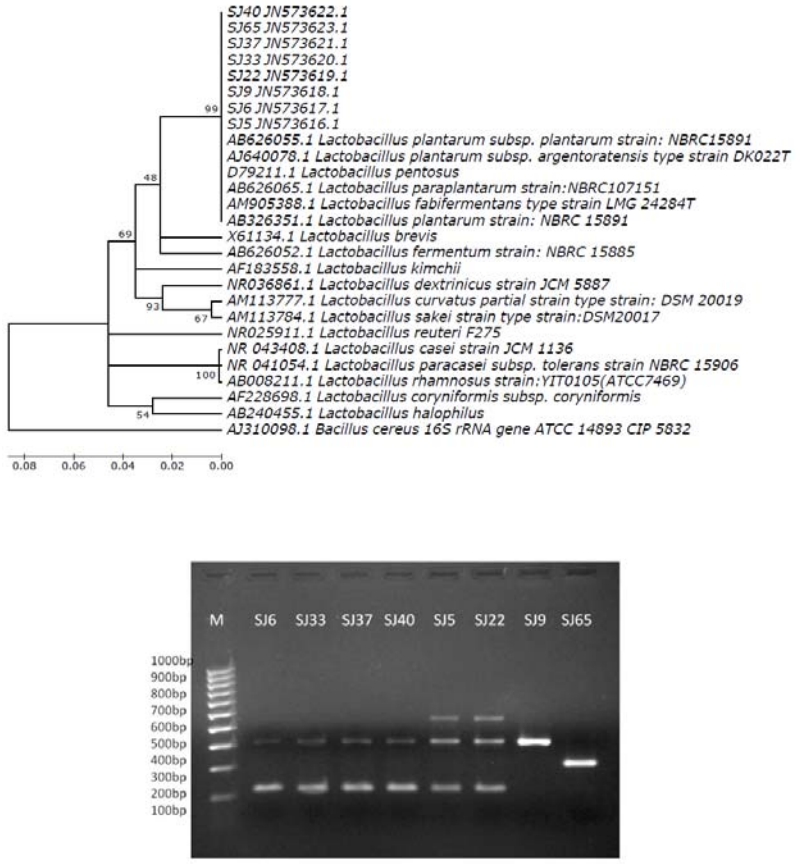

Fig. 2. The phylogenetic tree constructed for the eight isolates by Neighbour-Joining method and Multiplex PCR using species-specific primer Lane M is 100bp DNA ladder (D3687, Sigma). SJ6, SJ33, SJ37, SJ40 – *L. plantarum* ssp. *argentoratensis,* SJ5, SJ22 – *L. plantarum* but subspecies unidentified, SJ9 – *L. plantarum* ssp. *plantarum*, SJ65 – *L. pentosus*

RESULTS AND DISCUSSION

This is the first approach to our knowledge, in isolation of LAB from *Uttapam* batter (60 h), and was successful at isolating 46 bacilli. Eighteen potent isolates exhibiting wide spectrum of activity against various organisms lost its activity after protease treatment thus confirming bacteriocinogenic property. These organisms were clustered using RAPD as it seems to be rapid, reproducible and done at ease, as reported earlier[8]. RAPD of 18 bacteriocinogenic isolates showed bands ranging from 500 bp to more than 5 kb were grouped into 4 clusters. However in cluster 3, isolates SJ29 and SJ33 have minor variations from others in M13, were considered as a separate cluster (Fig. 1). Physiological and biochemical characteristics of isolates indicated they were non-motile, negative for catalase and arginine hydrolysis. They were homofermentative, indicating production of lactic acid as a major end product. Good growth was observed at 15, 37, and 45ºC, but no growth at 10ºC and 50ºC, indicating that they are mesophilic. Due to growth in various pH (4.5–9.5), all isolates were tolerant to both acidic and alkaline conditions. All isolates were tolerant at 4 and 6.5% salt concentrations, but not at 10%. All isolates utilized dextrose, maltose, fructose,

mannose, and esculin, while they did not utilize sugars like lactose, xylose, rafinose, melibiose, L & D arabinose, sodium gluconate, glycerol, glucosamine, dulcitol, inositol, sorbitol, mannitol, adonitol, rhamnose, xylitol, citrate, malonate, and sorbose. Isolates from each cluster exhibiting variation in their biochemical and physiological properties were represented in Table 2. Eight isolates that were chosen for 16S rRNA gene sequence analysis reveals 99% homology with *L. plantarum* and associated organisms Fig. 2. Several authors put forth that if sequence identity is greater than 97%, organisms cannot be differentiated effectively to subspecies level [9, 10]. Hence multiplex PCR using *recA* gene was utilized as a taxonomic marker to delineate closely related organisms[11, 12]. In this study multiplex PCR differentiated them wherein isolate SJ9 gave an amplification product at 318 bp belonging to *L. plantarum* ssp. *plantarum*; isolates (SJ6, SJ33, SJ37, and SJ40) gave two bands at 318 and 120 bp belonged to *L. plantarum* ssp. *argentoratensis*; isolate SJ65 gave a band at 218 bp belonging to *Lactobacillus pentosus* and isolates (SJ5 and SJ22) showed amplification products of 120, 318 and nearly 450 bp and closely associated with *L. plantarum* ssp. *argentoratensis*, however subspecies unidentified (Fig. 2). In conclusion, this is probably the first study reporting bacteriocinogenic lactobacilli to subspecies level showing broad spectrum of activity isolated from fermented sour *Uttapam* batter. These isolates may have a potential of being a probiotic and could be used for development of functional foods.

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