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Isolation and characterization of lactic acid bacteria from curd and cucumber

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Lactic acid bacteria (LAB) were isolated from curd and cucumber (*Cucumis sativus*) samples. The culture supernatants were screened for antimicrobial activity against LAB. Fourteen bacterial strains showing promising antimicrobial activity were further characterized by biochemical and molecular methods. These isolates were assigned to the genera *Lactobacillus, Pediococcus* and *Weissella,* on the basis of their morphological, biochemical, physiological characteristics, carbohydrate fermentation pattern and 16S rRNA gene sequences.

Keywords: Curd, cucumber, Lactic acid bacteria, ribotyping, biochemical characterization, bacteriocin

Introduction

Lactic acid bacteria (LAB) are a group of Gram-positive, non-spore forming, cocci or rod shaped, catalase-negative and fastidious organisms, considered as 'Generally Recognized as Safe' (GRAS) organisms. Mankind has exploited these bacteria for thousands of years for the production of fermented foods because of their ability to produce desirable changes in taste, flavour and texture. Different antimicrobial molecules such as lactic acid, acetic acid, hydrogen peroxide, carbon dioxide and bacteriocins produced by these bacteria are widely known to inhibit food borne pathogens and spoilage microorganisms, thereby extending the shelf-life and enhancing the safety of food products. Bacteriocins of LAB are extracellular bactericidal proteins that are secreted by the cells. On the basis of the protein structure, bacteriocins constitute a heterogeneous group of small peptides or high molecular weight proteins, or protein complexes. The inhibition spectrum of bacteriocins produced by LAB towards Gram-positive bacteria varies widely but is mostly confined to closely related species of the producing bacteria[1].

In recent years, much attention is being given to a large variety of bacteriocinogenic LAB from different sources, because their bacteriocins are considered to be safe in the form of food biopreservatives, since they can be degraded by gastrointestinal proteases[2,3]. Earlier reports indicate the isolation of LAB from grains, dairy and meat products, and fermenting vegetables as well as the mucosal surface of animals[4,5]. In the present investigation, we report the isolation and characterization of bacteriocin producing LAB from curd and cucumber (*Cucumis sativus*) samples.

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Materials and Methods

Chemicals and Microorganisms

Analytical grade chemicals and dyes were obtained from S R L India, while the DNA markers and bacteriological media were obtained from Sigma, USA and HiMedia Laboratories Pvt Ltd, India, respectively. The LAB (*Lactococcus diacetylactis*) used as an indicator organism was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India.

Isolation and Screening of LAB for Antimicrobial Activity

For isolating LAB, curd and cucumber samples were suspended, appropriately diluted in sterile normal saline, spread plated on de Mann Rogosa Sharpe (MRS) agar and incubated anaerobically at 37^{o}C for 3 d. The isolated colonies were transferred to MRS broth and purified by streaking twice on MRS agar. The individual bacterial colonies were stored in 0.8% MRS agar overlaid with 50% glycerol at –20^{o}C. All the colonies were ascertained for their ability to produce bacteriocins. Briefly, the cells were grown in MRS broth at 37^{o}C for 48 h and the supernatant was concentrated to1/10th of the original volume by flash evaporation. The concentrate was adjusted to *p*H 5.0 using 2N sodium hydroxide solution, sterilized by

passing through 0.22 µm membrane filter (Millipore, India) and the antimicrobial activity against *L. diacetylactis* was confirmed by agar well diffusion method[6].

Characterization of LAB

Overnight-incubated cultures of bacteriocin producing isolates were Gram stained and examined microscopically for morphology and phenotype. Catalase test was performed by adding few drops of 3% hydrogen peroxide to a test-tube containing 24 h-old culture of each isolate.

Biochemical Characterization

Growth of isolates was assessed in MRS broth at 15, 37 and 45°C and at *p*H 4.4, 7.0, 8.6 and 9.6 by incubating at 37°C. Salt tolerance was tested by incorporating 6.5, 10.0 and 15.0% (w/v) sodium chloride in MRS broth. Lactic acid and carbon dioxide production was tested in MRS broth containing inverted Durham's tube in the absence of citrate in the medium[7]. Production of ammonia in MRS broth containing 0.3% arginine and 0.2% sodium citrate replacing ammonium citrate was monitored using Nessler's reagent. Homo-fermentative and hetero-fermentative tests were carried out according to the method reported by Zuniga *et al*[8]. The ability of LAB to ferment various sugars was examined using HiCarbohydrate™ kit, HiMedia Laboratories Pvt. Ltd, India.

Molecular Characterization

DNA Extraction, 16S rRNA Gene Amplification and Sequencing

Genomic DNA from all the isolates was extracted as per the method of Delos Reyes Gavilan *et al*[9] with minor modifications. The DNA was extracted by phenol-chloroform method and precipitated with absolute alcohol. After air drying, the DNA pellets were dissolved in 10 m*M* Tris-1 m*M* EDTA (TE) buffer and analyzed by electrophoresis on 1% agarose gel containing 1 µg/mL ethidium bromide with Tris-acetate EDTA (TAE) buffer at 50 mA for 1 h.

The 16S rRNA gene sequence of LAB was amplified using universal primers of *E. coli* 16S rRNA gene sequence from 18-27 bp as forward primer (5′ -AGA GTT TGA TCC TGG CTC AG- 3′) and 1471-1492 bp as reverse primer (5' -TAC GGC TAC CTT GTT ACG ACT T- 3') as described by Pandey *et al*[10]. The reaction was carried out in Eppendorf Master Cycler. The PCR conditions were standardized as follows: initial denaturation at 94°C for 5 min, 30 cycles of denaturation of 94°C for 30 sec, annealing at 54°C for 30 sec and extension at 72°C for 90 sec and final extension at 72°C for 5 min.

The PCR products were analyzed by 1% agarose gel electrophoresis with 1 µg/mL ethidium bromide and visualized with hand held UV trans-illuminator. The 16S rDNA amplicon in the gel was excised and purified from agarose using Gel extraction kit (Advanced Micro Device Pvt Ltd, India) as per the manufacturers instructions. The purified DNA was sequenced using ABI Sequencer Model 3700.

The 16S rRNA gene sequences determined (ca. 600-700) were aligned along with the sequences of the type strains obtained from the GeneBank using the CLUSTAL_X program version 1.82[11]. Distance matrices for aligned sequences were calculated by the two-parameter method[12]. A phylogenetic tree was constructed by neighbor-joining method[13] with the programme PHY-LIP (version 3.64) available at.http://evolution.genetics.washington.edu/phylip.html. Confidence values of individual branches in phylogenetic tree were determined by bootstrap analysis based on 1000 samplings Felsenstein[14].

Antibiogram of LAB

Seventy-five microlitre of overnight grown culture of each isolate was spread plated on MRS agar plates and Octa discs of HiMedia Laboratories Pvt. Ltd, India, were placed upside down, pressed on the top of agar plates and incubated overnight at 37°C. Bacterial resistance was defined as the absence of growth inhibition zone around the antibiotic discs.

Results and Discussion

All the 50 colonies isolated from curd and cucumber samples were found to be Gram-positive and catalase-negative, but only 14 isolates showing potent antimicrobial activity against *L. diacetylactis* were chosen for further characterization (Fig. 1). Morphologically, the cells of 5 isolates were coccus type and arranged either in pairs or tetrads. Their colonies on MRS agar were circular, low convex with entire margin and cream coloured. The cells of remaining 9 rod shaped isolates were arranged in pairs or chains. Their colonies on MRS agar were found to be circular, low convex with entire margin and non-pigmented.

Physiological and Biochemical Characteristics

Isolates were divided into two major groups, Group A and B, based on the cell shape. Group A consisted

of 5 sphere shaped isolates which were further sub-divided into 2 subgroups according to their growth pattern at different temperatures and carbohydrate utilization pattern. Subgroup A1 grew at 45°C and utilized maltose, whereas the subgroup A2 grew at 10°C without utilizing maltose for acid production.

Group B, consisting of 9 rod shaped isolates was further divided into 2 subgroups, Subgroup B1 and B2 according to their size. Subgroup B1 consisted of 5 short rods and subgroup B2 consisted of 4 long rod shaped bacteria. Subgroup B1 was further sub-divided into Sub-subgroups B1a and B1b, based on the production of ammonia from arginine. Only subgroup B1a produced ammonia. Subgroup B2 was further divided into Sub-subgroups B2a and B2b based on biochemical tests and sugar utilization pattern. The distribution of isolates among the groups and their general characters are shown in Table 1 while the characteristics regarding their carbohydrates utilization are shown in Table 2.

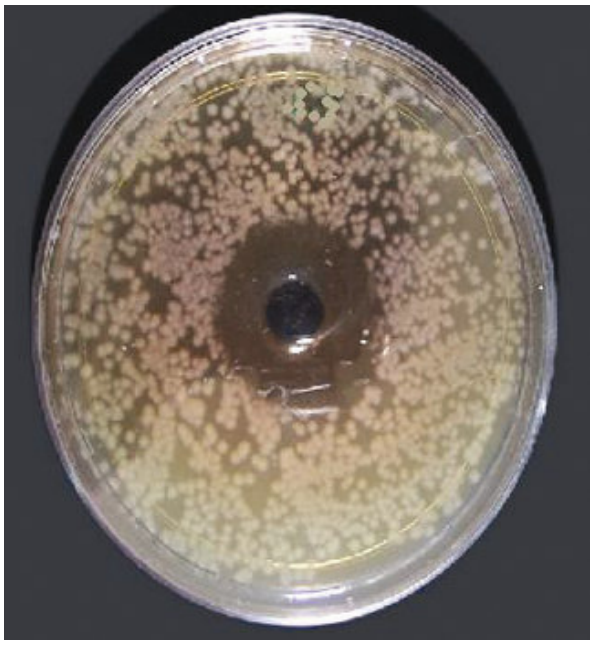

Fig. 1—Inhibition of *L. diacetylactis,* an indicator organism by cell free supernatant concentrate of LAB isolate.

Table 1—Morphological and biochemical properties of bacteriocin producing LAB

Characteristics		Group A		Group B			
				Subgroup B1		Subgroup B2	
		Subgroup A1 *P. acidilactici* (4 isolates)	Subgroup A2 *P. pentosaceous* (1 isolate)	Sub-subgroup B1a *W. cibaria* (2 isolates)	Sub-subgroup B1b *W. paramesenteroides* (3 isolates)	Sub-subgroup B2a *L fermentum* (2 isolates)	Sub-subgroup B2b L plantarum (2 isolates)
Cell shape		Cocci		Short rods		Long rods	
Cell arrangement		Pairs and tetrads		Single, pairs or chains			
Catalase		-ve	-ve	-ve	-ve	-ve	-ve
Acid production from Glucose		+	+	+	+	+	+ve
Gas production from glucose		-	-	+	+	+	-ve
NH_3 production from arginine		+	+	+	-	+	-ve
HHD Test		Homo	Homo	Hetero	Hetero	Hetero	Homo
Growth at temp	15°C	-	+	-	-	+	+
	37°C	++	++	++	++	++	++
	45°C	+	-	+	-	+	-
Growth at *p*H	3.9	W+	W+	-	-	-	-
	4.4	W+	W+	-	-	-	-
	8.6	+	+	+	+	+	+
	9.6	+	+	-	-	+	+
Growth at NaCl	6.5%	W+	W+	+	+	-	W+
	10%	-	-	-	-	-	-
	15%	-	-	-	-	-	-

Legend: growth (+), no growth (-), luxurious growth (++), weak growth (W+), homo-fermentative (Homo), hetero-fermentative (Hetero), Homo-hetero fermentative differentiation (HHD).

Table 2—Carbohydrate utilization pattern of bacteriocin producing LAB

Carbohydrates	Group A		Group B			
			Subgroup B1		Subgroup B2	
	Subgroup A1 *P. acidilactici* (4 isolates)	Subgroup A2 *P. pentosaceous* (1 isolate)	Sub-subgroup B1a *W. cibaria* (2 isolates)	Sub-subgroup B1b *W. paramesenteroides* (3 isolates)	Sub-subgroup B2a *L. fermentum* (2isolates)	Sub-subgroup B2b *L. plantarum* (2 isolates)
Lactose	-	-	-	-	+	+
Xylose	+	W+	+	-	+	-
Maltose	-	+	-	+	+	+
Fructose	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+
Galactose	+	+	-	-	+	+
Raffinose	-	-	-	-	+	+
Trehalose	+	+	-	+	+	+
Mellibiose	-	+	-	+	+	W+
Sucrose	+	+	+	+	+	+
L Arabinose	+	+	+	+	-	-
Mannose	+	+	+	W+	+	+
Inulin	-	-	-	-	-	+
Sodium gluconate	-	-	-	+	-	+
Salicin	+	+	+	-	-	+
Glucosamine	+	-	W+	W+	-	-
Ribose	-	-	-	-	-	+
Cellobiose	+	+	+	-	-	+
Esculin	+	+	+	+	-	+

Legend: positive (+), negative (-), weak reaction (W+).

Molecular Identification of Bacteriocin producing LAB

Sequencing results of 16S rRNA was exported to the database and checked for homology alignment. Based on the alignment results, Group A1 was found as *Pediococcus acidilactici*, Group A2 as *P. pentosaceous*, Group B1a as *Weissella cibaria*, Group B1b as *W. paramesenteroides* Group B2a as *Lactobacillus fermentum* and Group B2b as *L. plantarum.* The 16S rDNA sequences of representative strains of each group have been submitted to EMBL database; the accession numbers given were: FJ390107 for *P. acidilactici,* FJ390108 for *P. pentosaceous*, FJ390109 for *W. cibaria,* FJ390110 for *L. fermentum,* FJ390111 for *L. plantarum* and FJ390112 for *W. paramesenteroides*.

Subgroup A1 contained 4 isolates whereas subgroup A2 only 1. All the isolates were sphere shaped, produced ammonia from arginine, homo-fermentative, catalase-negative and did not produce carbon dioxide from glucose. *P. acidilactici* and *P. pentosaceous* were able to hydrolyze arginine as reported earlier[15]. Good growth was observed in all the strains at 37°C while there was no growth at 45°C in the case of subgroup A2 isolates. *P. acidilactici* could arbitrarily be differentiated from *P. pentosaceous* by its ability to grow at 50°C and survive even at 70°C for 10 min[15,16]. All the 5 isolates were able to utilize carbohydrates like trehalose, dextrose, fructose and esculin. Ability to ferment maltose was a special character of *P. pentosaceous*[16]. On the basis of these key characters, subgroup A1 isolates were identified as *P. acidilactici* and subgroup A2 isolate as *P. pentosaceous*. Based on the 16S rRNA gene sequence analysis, subgroup A1 isolates represented 99% similarity with *P. acidilactici* strain BFE 846 and subgroup A2 isolates represented 99% similarity with *P. pentosaceous*.

Subgroup B1 contained 5 isolates, which were further sub-divided into 2 sub-subgroups according to production of ammonia from arginine. sub-subgroup B1a contained two isolates whereas sub-subgroup

B1b contained 3 isolates. All the 5 isolates were heterofermentative, rod shaped, catalase-negative and grew at 6.5% sodium chloride. These are the major characters of *Weissella*[17]. *W. cibaria* differed from majority of *Weissella* by being able to grow at 45°C and producing ammonia from arginine. Based on the 16S rRNA gene sequences analysis, isolates of Sub-subgroup B1a represented 99% similarity with *W. cibaria* and isolates of sub-subgroup B1b represented 99 % similarity with *W. paramesenteroides.*

Subgroup B2 contained 4 isolates, which were further sub-divided into two sub-subgroups based on the biochemical tests and sugar utilization pattern. Sub-subgroup B2a had 2 rod shaped isolates, which were heterofermentative, catalase-negative, and grew at 45°C, produced carbon dioxide from glucose and ammonia from arginine. Both isolates utilized carbohydrates like lactose, xylose, maltose, raffinose, melezitose, rhamnose, salicin and sorbose. Similar characters for *L. fermentum* have been observed previously by Chantaraporn & Somboon[18]. The 16S rRNA analysis of two isolates showed 99% similarity to *L. fermentum.* Sub-subgroup B2b contained two rod shaped, catalase negative, homofermentative isolates, which did not produce carbon dioxide from glucose and ammonia from arginine. Among the carbohydrates examined, both isolates fermented lactose, maltose, mannitol, mannose, raffinose, ribose, salicin, esculin, galactose and trehalose. These are the common characters of *L. plantarum* as reported earlier by Kandler & Wiss[19]. The 16S rRNA analysis of two isolates showed 99% similarity with *L. plantarum.* Fig. 2 shows the phylogenetic tree based on 16S rRNA sequences of representative strains of each group.

The antibiogram of one isolate from each subgroup was studied using Octa discs. The behaviour of each isolate to different antibiotics in terms of sensitivity and resistance has been shown in Table 3. All the isolates were found sensitive to most of the broad-spectrum antibiotics and resistant to antibiotics active on Gram-negative bacteria.

These bacteriocin-producing isolates have been anticipated to have enormous potential for food application as biopreservatives. Detailed studies on the production, purification and characterization of bacteriocins from these isolates are in progress.

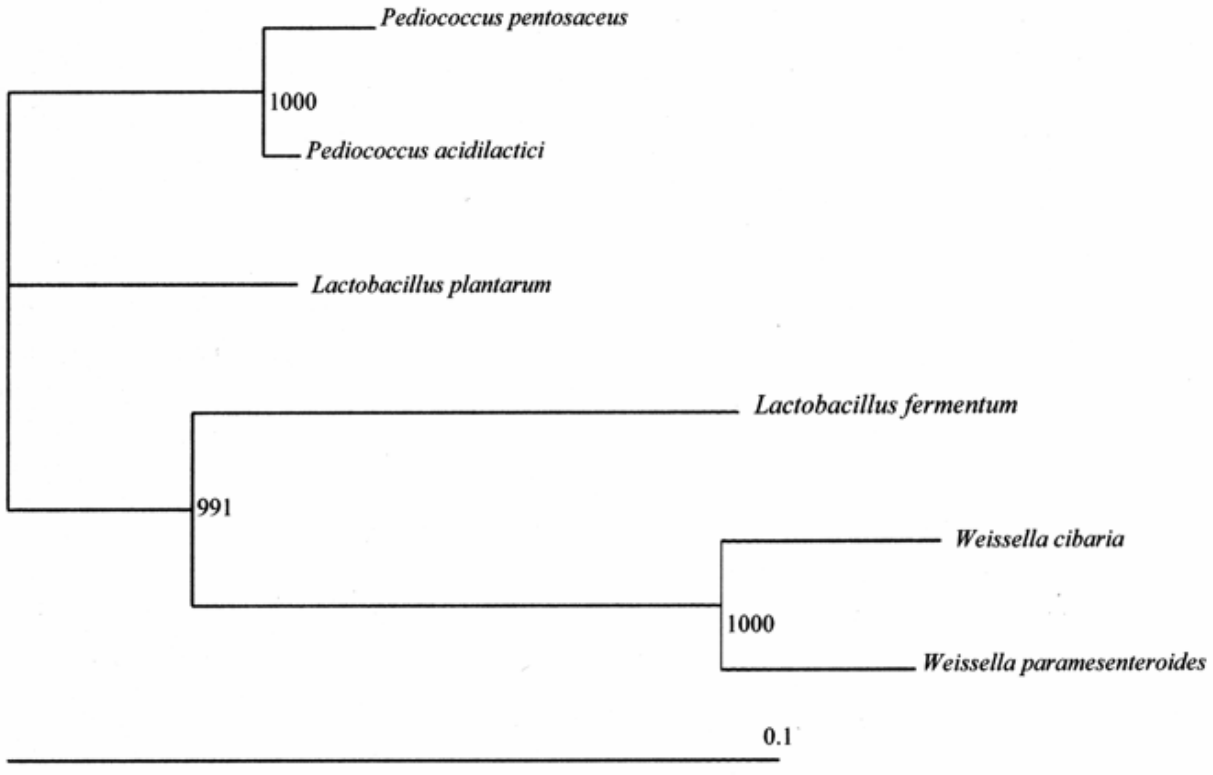

Fig. 2—Phylogenetic relationships based on 16S rDNA sequences of curd and cucumber isolates.

Table 3—Antibiogram of bacteriocin producing LAB

Antibiotics	Group A		Group B			
			Subgroup B1		Subgroup B2	
	Subgroup A1 *P. acidilactici* (4 isolates)	Subgroup A2 *P. pentosaceous* (1 isolate)	Sub-subgroup B1a *W. cibaria* (2 isolates)	Sub-subgroup B1b *W. paramesenteroides* (3 isolates)	Sub-subgroup B2a *L fermentum* (2 isolates)	Sub-subgroup B2b *L. plantarum* (2 isolates)
Nalidixic acid 35µg	R	I	R	R	R	R
Norfloxacin 10µg	R	R	S	I	R	R
Co-trimaxazole 25µg	R	R	R	R	R	R
Gentamycin 10µg	S	I	S	S	R	S
Ampicillin 25µg	S	S	S	S	S	S
Mecillanam 33µg	S	S	S	S	S	S
Lomefloxacin 10µg	S	S	R	R	R	ND
Azithromycin1 5µg	S	S	S	S	S	ND
Vancomycin 30µg	R	S	S	S	R	ND
Doxycycline HCl 30µg	S	S	S	S	S	ND
Nitrofurantoin 300µg	S	I	R	R	S	S
Gatifloxacin 5µg	S	S	S	S	S	ND
Amoxycillin 10µg	S	S	S	S	S	S
Cloxacillin 5µg	R	S	S	S	R	S
Erythromycin 10µg	S	S	S	S	S	S
Tetracycline 10µg	S	S	S	S	S	S
Cephalexin 30µg	R	R	R	R	R	ND
Penicillin G 2U	S	S	S	S	S	S
Cephadrine 30µg	S	S	S	S	R	ND
Lincomycin 10µg	S	S	S	S	S	S
Cefuroxime 30µg	S	S	S	S	S	S
Cephaloridine 30µg	S	S	S	S	S	S
Kanamycin 30µg	R	R	R	R	R	ND
Methicillin 5µg	R	R	S	S	S	ND

Legend: sensitive (S), resistant (R), intermediate (I), not determined (ND)

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