

Isolation and Screening of *Lactobacillus* bacteria for Ability to Produce Antibiotics

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ABSTRACT

Antibiotic is one of the important commercially exploited secondary metabolites produced by bacteria and used in a wide range. Most of the antibiotics used today are isolated from the microbes. Bacteria are easy to culture, isolate, maintain and to improve their strain. Isolation of lactic acid bacteria (LAB) from soil, yoghurt and cheese was carried out. LAB were cultivated on De Man Rogosa Sharpe (MRS) agar and were characterized based on colony morphology, cell shape and biochemical tests. Out of eight samples analyzed, 96 isolates were identified as LAB. The bacterial isolates were identified as *Lactococcus Lactis*, *Lactobacillus Brevis*, *Lactobacillus casei*, *Pediococcus damnosus*, *Lactobacillus rhamnosus*, *Lactobacillus Plantarum*, *Lactobacillus pentosus*, *Enterococcus faecalis*, *Staphylococcus Simulans*. Using MRS broth, the isolated LAB were screened for production of bacteriocins. Further, 96 LAB screened for bacteriocin production, 12 LAB were identified as bacteriocin producers. Out of 12 LAB, *Enterococcus faecium* (CST-1) was identified as potential bacteriocin producer against *Bacillus subtilis* MTCC-10403 *Pseudomonas aureginosa* MTCC-4676 microorganisms used while *Pediococcus damnosus* had the least bacteriocin activity against *Staphylococcus aureus* and *Bacillus subtilis*.

Keywords: *Pseudomonas aureginosa*; *Pediococcus damnosus*; bacteriocin activity; *Staphylococcus aureus*; *Bacillus subtilis*

1. INTRODUCTION

The term Lactic Acid Bacteria (LAB) was gradually accepted in the beginning of the 20th century (Carol *et. al.*, 2010). Lactic acid bacteria is having direct influence on the organoleptic, shelf-life and nutritional characteristics because of this phenomenon LAB has long history of application in fermented foods (Leroy F *et.al.*, 2004). Lactic acid bacteria is generally gram positive, non sporulating, usually non motile, catalase negative, non respiring cocci or rods, and fastidious organisms, LAB produce lactic acid as an end product through fermentation of carbohydrates. Lactic acid bacteria is generally classified based on morphology, range of sugar utilization and mode of glucose fermentation. Lactic acid bacteria consisting of different number of bacterial genera within the phylum Firmicutes.

The genera includes *Lactobacillus*, *Leuconostoc*, *pediococcus*, *streptococcus*, *Enterococcus*, *Oenococcus*, *Lactococcus*, *Carnobacterium*, *Lactosphaera*, *Vagococcus*, *Melliococcus*, *Tetragenococcus* and *weissels* are recognized as LAB (Stiles and Holzapfel, 1997; Holzapfel *et al.*, 2001; Jay, 2000).

Lactic acid bacteria lack the ability to synthesize cytochromes and porphyrins and therefore cannot generate ATP, they obtain ATP only through sugar fermentation since lactic acid bacteria does not use oxygen in their energy production. Lactic acid bacteria can be easily grown in aerobic and as well as anerobic conditions, they are protected by oxygen byproducts because they have peroxidases, they are also known as aerotolerant anaerobes (Michaela *et al.*, 2009). These bacteria are differentiated from the other micro organisms by the ability to convert hexoses to lactic acid.

Lactic acid bacteria can be generally divided into two groups based on the sugar fermentation pathways, those organisms which produce exclusively lactic acid as end product are termed as Homofermentative lactic acid bacteria, and organisms which produce acetic acid, ethanol, CO₂ in addition to lactic acid are termed as Heterofermentative LAB. All members of *Pediococcus*, *Lactococcus*, *Streptococcus*, *Vagococcus*, along with some lactobacilli are homofermenters.

Carnobacterium, *Oenococcus*, *Enterococcus*, *Lactosphaera*, *Weissells* and *Leconostoc* and some Lactobacilli come under heterofermenters. The heterofermentative LAB gain more importance in food industries by producing aromatic and flavour components such as diacetyl and acetaldehyde.

LAB is Generally Recognized as safe (GRAS) organisms, Lactic acid bacteria is generally used in the production of fermented foods because for their ability to produce desirable change in colour, texture, taste and flavour. These lactic acid bacteria are mostly known to inhibit food borne pathogens and food spoilage microorganisms by producing different antimicrobial substances such as bacteriocins, lactic acid, ethanol, acetic acid, hydrogen peroxide and CO₂ and several aromatic compounds, because of these characteristic features LAB generally utilized for enhancing the safety and extending shelf-life of food products. Bacteriocins produced by LAB are nothing but extracellular bacterial proteins secreted by the cells. The Bacteriocins of LAB can be easily degraded by the human gastrointestinal proteases because of this important feature LAB generally gained importance in the recent years in food preservation (Cleveland *et al.*, 2001).

In recent years the use of Lactic acid bacteria as "Probiotics" is gaining more importance since because mainly lactobacillus and bifidobacteria may have several therapeutic functions (Berg, 1996; Oberg *et al.*, 1998).

The objective of the present study was to isolate lactic acid bacteria from different sources like soil, curd, cheese and to screen these isolates for production of antimicrobial compounds.

2. MATERIALS AND METHODS

API 50CHL identification kit was purchased from Biomerieux, USA. MRS HiVeg agar, MRS HiVeg broth was procured from Himedia, Mumbai. All other chemical used in the present study were of analytical grade and purchased from Himedia, Mumbai, India and Merck, Bangalore, India.

2. 1. Collection of samples

A total number of eight samples were analyzed for isolation and screening of LAB. Out of eight samples, four were soil samples were collected with sterile spoons from milk waste dumping yard at Visakhapatnam, tirupati and hyderabad, India at a depth of 20 cm and saved into clean bags. Curd and cheese samples were collected from chennai local market,

Tamilnadu and were brought to the laboratory under sterile conditions. One gram of each collected soil, curd and cheese sample was serially diluted upto 10^{-1} to 10^{-7} in 0.9% NaCl solution and plated on MRS agar medium, incubated at 37 °C for 24 hours.

2. 2. Isolation and identification of the isolates

From each plate of MRS agar, colonies with different morphologies were picked up randomly and transferred into the tubes containing MRS broth and were incubated in aerobic conditions at 37°C for 24 hours. This procedure was repeated until purity of the culture was established on the basis of colony appearance and microscopic observations such as gram staining. Each of the isolates was first tested for catalase by placing a drop of 3% hydrogen peroxide solution on the cells. Immediate formation of bubbles indicated the presence of catalase in the cells. Only those isolates which were catalase- negative were Gram-stained, and only those which were Gram-positive were further identified by the API 50CHL identification kit (Biomerieux).

The biochemical tests performed were Simmon's Citrate Slant test, Indole test, Methyl Red (MR), Voges Proskauer (VP) and Oxidase tests. Identification of isolates obtained in pure culture was based on Gram staining, morphology, growth characteristics on selective and differential media such as MRS agar and biochemical test results recommended in the Bergey's Manual of Determinative Bacteriology.

2. 3. Biochemical characterization of the isolates

2. 3. 1. Carbohydrate utilization

The isolated LAB was further identified by their fermentative ability of carbohydrates using API50CHL kit.

2. 3. 2. Sodium chloride (NaCl) tolerance

For determination of NaCl tolerance, all the isolates were grown in MRS broth supplemented with different concentrations of NaCl (1-9 %). The broths were inoculated with 100 µl overnight culture of the isolates and incubated anaerobically at 37 °C for 24 hour. After 24 hour incubation, growth was determined using a spectrophotometer, reading the optical density at 600 nm.

2. 3. 3. Optimization of culture temperature

For the determination of optimum culture temperature growth of the isolates, 100 µl overnight culture of the isolates were inoculated into MRS broth with varying temperatures ranging from 20, 25, 30, 35, 40 and 40 °C. The inoculated broths were then incubated for 24 h. Growth of the bacterial isolates was measured using a spectrophotometer at 560 nm.

2. 3. 4. Optimization of culture pH

For the determination of optimum culture pH growth of the isolates, 100 µl overnight culture of the isolates were inoculated into MRS broth with varying pH ranging from 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. The inoculated broths were then incubated for 24 h at 37 °C. Growth of the bacterial isolates was measured using a spectrophotometer at 560 nm.

2. 3. 5. Antibacterial activity of the isolates

Agar well diffusion method was used to determine the antimicrobial activities of the isolated *Lactobacillus* sp according to the method of Murray et al., 1995. Four different indicator microorganisms belonging to gram positive and gram negative groups such as *Bacillus subtilis* (MTCC), *Staphylococcus aureus* (MTCC-3160), *Escherichia coli* (MTCC-1652), *Pseudomonas aeruginosa* (MTCC-4676) were used in this study as test pathogen.

3. RESULTS

3. 1. Growth and morphology

A total of 96 isolates were identified as *Lactobacillus* species using MRS agar medium. The isolates were subjected to grow on selective MRS agar media and produced round shape, off-white to cream color, shiny colonies those were quite similar to the reference *Lactobacillus* spp. grown on MRS agar media (Figure 1A). Isolates when Gram stained, found rod shaped, short-medium chain and positive in Gram reaction those all are typical characteristics of *Lactobacillus* spp. (Figure 1B). The isolates were able to grow at pH between 5.0 and 7.0, but the optimum growth was observed at pH between 5.5 and 6.5 when grown in MRS broth at 37 °C.

3. 2. Biochemical characterization

For biochemical characterization, catalase, oxidase, indole, MR, VP and citrate tests were conducted and found negative for all the isolates. Carbohydrate utilization test was performed by using API50CHL identification kit (Biomérieux) to investigate whether the isolates can ferment carbohydrates and the results were described in Table No.1.

3. 3. Determination of antimicrobial activity

The crude extract of eight strains of lactic acid bacteria gave zones of inhibition on to the indicator organisms. The diameters of inhibition are included between 2mm to 6mm. the biggest diameter of 6mm was obtained with crude extract of CST-1 against *Bacillus subtilis* (Figure 2) and the smallest diameter of 1mm was obtained with crude extract of CST-6 against *E.coli*.



Figure 1. Strain CST-1 *Enterococcus faecium* on MRS agar.

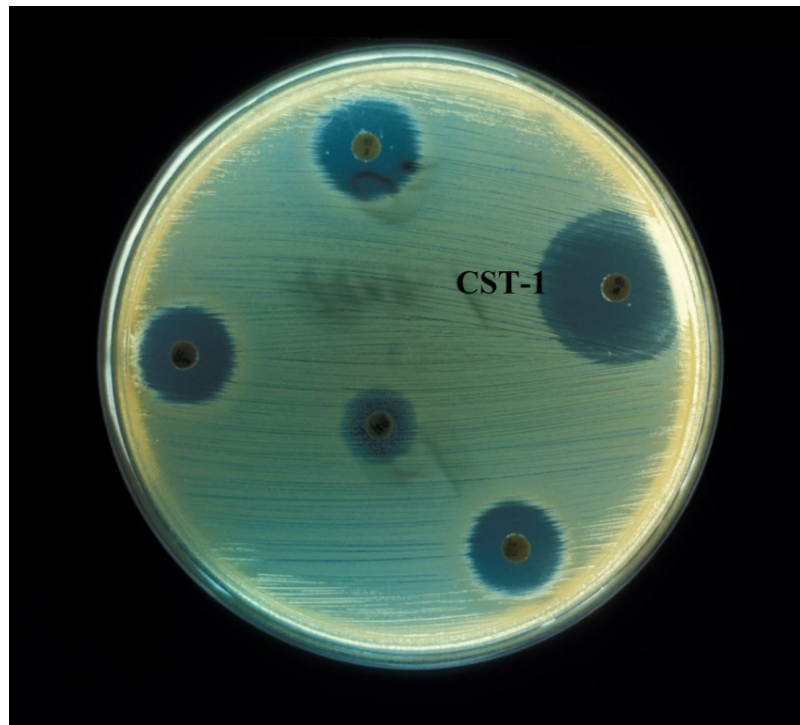


Figure 2. Well diffusion method for measuring the zone of inhibition.



Figure 3. API 50 CHE strip analysis for strain CST-1.
 Yellow indicates positive.
 Blue indicates negative.
 Green indicates positive/negative.

Table 1. Isolation and the identification of isolates.

Strain code	Sampling date	Source	Location	Species
CST-1	15/10/2012	soil from visakha diary	visakhaptanam	<i>Enterococcus faecium</i>
CST-2	02/11/2012	soil from vijaya diary	Tirupathi	<i>Lactobacillus. Brevis</i> <i>Lactobacillus casei</i>
CST-3	03/11/2012	soil from visakha diary	visakhaptanam	<i>Pediococcus damnosus</i> <i>Lactobacillus rhamnosus</i>
CST-4	15/11/2012	curd sample	Chennai local market	<i>Lactococcus lactis</i>
CST- 5	19/12/2012	soil from tirumala dairy	Hyderabad	<i>Lactobacillus Plantarum,</i> <i>Lactobacillus pentosus.</i>
CST-6	16/01/2013	cheese	Chennai local market	<i>Enterococcus faecalis</i>
CST-7	21/01/2013	curd sample	Chennai local market	<i>Staphylococcus Simulans</i>
CST-8	06/02/2013	cheese	Chennai local market	<i>Enterococcus Feacalis</i>

Table 2. Zone of Inhibition shown by isolated species on different test pathogens.

Isolated colonies	<i>Bacillus subtilis</i> (MTCC-10403)	<i>Staphylococcus aureus</i> (MTCC-3160)	<i>Escherichia coli</i> (MTCC-1652)	<i>Pseudomonas aeruginosa</i> (MTCC-4676)
<i>Enterococcus faecalis</i>	5.5mm	3.8mm	5.4mm	6.2mm
<i>Lactobacillus. Brevis</i>	6.2±0.4mm	4.5mm	3.8mm	6.4mm
<i>Pediococcus damnosus</i>	5±0.4mm	3.8 mm	4.2 mm	5.4 mm
<i>Lactococcus lactis</i>	5.8 mm	4.2 mm	5.4 mm	9 mm
<i>Lactobacillus Plantarum.</i>	6.2±0.4mm	4.4 mm	5.4 mm	7.2mm
<i>Enterococcus faecium</i> CST-1	10.2±0.4mm	8.4 mm	6.8mm	8.6mm
<i>Lactobacillus pentosus</i>	6.2±0.4mm	3.8 mm	4.2 mm	5.4 mm
<i>Staphylococcus Simulans</i>	3.8 mm	4.2 mm	5.4 mm	10mm
<i>Lactobacillus rhamnosus</i>	4.4 mm	3.6 mm	4.2 mm	5.4 mm
<i>Lactobacillus casei</i>	6.8 mm	4.4 mm	5.4 mm	7.2mm

Table 3. Biochemical characterization of isolated strains using API 50 CHE strip.

ISOLATES	CST-1	CST-2	CST-3	CST-4	CST -5	CST -6	CST -7	CST-8
Control	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-
L-Arabinose	+	+	-	+	+	-	+	-
D-Ribose	+	+	-	+	+	-	+	+
D-xylose	+	+	-	-	-	+	+	-
L-Xylose	-	-	-	-	-	-	-	-
D-Adonitol	-	-	-	-	-	-	-	-
Methyl-βD-xylopyranoside	-	-	-	-	-	-	-	-
D-Galactose	+	+	-	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	?	+
D-Mannose	+	+	+	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	?	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-
D-Mannitol	-	+	-	+	+	+	-	+
D-Sorbitol	-	-	-	-	+	+	+	-

Methyl- α D-Mannopyranoside	+	-	-	+	+	-	+	+
Methyl- α D-Glucopyranoside	-	-	-	-	+	-	-	-
NAcetylglucosamine	+	+	+	+	+	+	+	+
Amygdalin	+	+	-	-	+	-	?	+
Arbutin	+	+	-	+	+	+	+	-
Esculinferric citrate	-	-	-	-	-	-	-	-
Salicin	-	+	-	+	+	+	-	?
D-Cellobiose	+	+	-	+	+	-		+
D-Maltose	+	+	-	+	+	-	+	+
D-lactose(bovine origin)	-	+	-	+	+	-	+	-
D-Melibiose	-	+	-	?	+	+	+	-
D-Saccharose	+	+	-	+	+	-	+	+
D-Trehalose	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-
D-Melezitose	-	-	-	-	+	-	-	-
D-Raffinose	-	-	-	-	+	-	-	-
Starch	-	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-
Genitobiose	-	-	-	+	+	-	+	+
D-Turanose	-	+	-	-	-	-	?	-
D-Lyxose	-	-	-	-	-	-	-	-
D-tagatose	+	+	-	+	-	+	-	+
D-Fucose	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-
D-Arabitol	-	?	-	-	-	?	-	-
L-Arabitol	-	-	-	-	-	-	-	-
Potassium gluconate	-	+	-	-	-	-	?	-
Potassium 2-ketogluconate	-	-	-	-	-	-	-	-
Potassium 5 ketogluconate	-	-	-	-	-	-	-	?

4. DISCUSSIONS

Antimicrobial activity is one of the most important selection criteria for probiotics. Antimicrobial effects of lactic acid bacteria are incurred by producing some substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins. All the *Lactobacillus* isolates showed to inhibit the test organisms included in this study though they vary in zone of inhibition diameter.

5. CONCLUSION

The cell-free supernatants from eight strains of lactic acid bacteria exhibited antimicrobial activity. Most of Lacto bacillus was grown in between pH 5.5 and 6.5 at a temperature of 37 °C. The potential application of secondary metabolites as consumer friendly bio preservatives either the form of protective cultures are as additives is significant besides being less potentially toxic or carcinogenic than current antimicrobial agents, lactic acid bacteria and their byproducts have been shown to be more effective and flexible in several applications. The further studies will be focused on the optimization and characterization of these antibacterial compounds.

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