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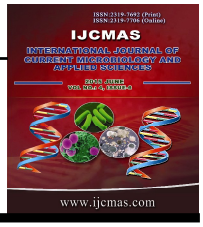

Original Research Article

Isolation, Identification and Analysis of Probiotic Properties of *Lactobacillus* Spp. from Selected Regional Dairy Product

Ankita Chakraborty and Jayati Bhowal*

School of Community Scienceand Technology, Indian Institute of Engineering Science and Technology, Shibpur, (Formerly Bengal Engineering and Science University, Shibpur), Howrah -711103, West Bengal, India
**Corresponding author*

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Lactic Acid Bacteria, *Lactobacillus*, Fermented Milk, Antimicrobial Activity, Antibiotic Resistance

A B S T R A C T

Lactic acid bacteria were isolated from the traditional food named dahi in West Bengal, India. The isolates were assigned to the genera *Lactobacillus* on the basis of their morphological, physiological and some biochemical characteristics. It was observed that the isolated *Lactobacillus* species were resistant to inhibitory substances like phenol (0.4%) and NaCl (4%). Good growth was observed both in acidic and alkaline conditions, while maximum growth was observed at pH 5.5-6.5. Isolated *Lactobacilli* were able to ferment some carbohydrates and produced organic acid from skim milk. The isolates were resistant to all the selected antibiotics used in this study. Cell free supernatant obtained from the isolates exhibited inhibitory activity against selected pathogens of both Gram positive and Gram negative group.

Introduction

Lactic acid bacteria (LAB) have attained major attention for their widespread use in the production of fermented foods (Farnwarth, 2005) which are characterized by hygienic safety, better organoleptic properties and perhaps the probiotic qualities (Savadogo *et al.*, 2006). LAB are used as starter culture in fermentation and some of them are also natural component of intestinal micro flora (Fuller, 1992), (Holzapfel *et al*., 2001). *Lactobacilli* are one of the most important genera of LAB (Coeuretetal., 2003)and have tremendous industrial application (Stiles,1996).Lactic acid bacteria especially *Lactobacilli* and *Bifidobacteria* are the most common bacteria considered as potential prebiotics (Espirito Santo *et al*., 2003). Various strains of *Lactobacilli* are used as health-promoting probiotic ingredients since they have several therapeutic functions (Oberg *et al.*, 1998) including antibiotic resistance (Curragh and Collins, 1992), bile tolerance (Walker and Gilliland,1983) and gastric juice tolerance (Kilara, 1982). *Lactobacilli* comprise a large and diverse group of Gram positive, non-spore forming, catalase negative, and rod shaped bacteria able to produce lactic acid as the main end product of the fermentation of carbohydrates (Pelinescu *et al*., 2009).

Different *Lactobacillus* species are non-pathogenic and do not produce toxic substances. In recent years much attention is being given to isolation of *Lctobacilli* from different sources which are also used as bio preservatives traditional fermented milk product and is a very popular menu at the end of the meal in India subcontinent. Dahi is manufactured from milk by traditional method using LAB as indigenous starter culture. However, very little information is available on the characteristics of *Lactobacillus* microflora present in locally available dahi. In order to provide health benefits by *Lactobacilli* present in dahi, they require their relevant characterization and identification. The present study has been carried out with objective to screen *Lactobacilli* from locally available dahiand to study probiotic properties with the ultimate objective of its potential use in probiotic yogurt preparation.

Material and Methods

All chemicals and dyes used in this study were of analytical grade, purchased from Merck, India. The bacteriological media were obtained from HiMedia Laboratories Pvt. Ltd., India. The traditional curd sample for isolation of *Lactobacillus* was purchased from local market of Kolkata, India and kept in sterile plastic container. Immediately after collection the sample was stored aseptically in low temperature (4^0C) for the isolation of *Lactobacillus*. Six different antibiotics were obtained from local medicinal shop. Various Gram positive i) *Staphylococcus auras*, ii) *Bacillus subtilis* and Gram negative bacterial strains i) *E. coli*. and ii) *Pseudomonas aeruginosa* were provided by National Cholera Institute, Kolkata and used for in vitro antimicrobial study. These Gram positive and Gram negative test organisms were maintained in Brain Heart Infusion Agar butt-slants in screw-capped tubes and kept at 4^oC.The isolation was performed in School of Community Science and Technology, Indian Institute of Engineering, Science and Technology, Shibpur, Howrah, India.

Isolation of *Lactobacillus*

Lactobacillus was isolated from locally available dahi by using de Mann Rogosa Sharpe (MRS) agar media(De Man *et al*., 1960).1 gm of curd sample was mixed with 9 ml of sterile phosphate buffer saline (PBS), homogenized gently, serially diluted 10 fold in PBS and pour plated aseptically on MRS agar media. Plates were incubated at 37^oC for 48 hrs in anaerobic condition. Colonies differ in morphology, pigmentation; shape and size were subcultured in MRS broth. Initially all of the isolates were examined for Gram staining and catalase production. Only the Gram-positive, catalase-negative and rod shape isolates were then purified by streak plating using the same medium. After several subcultures, finally the single colony of *Lactobacillus* was isolated by observing their colony morphology and some biochemical tests (Gram staining, catalase, endospore and motility test) and the culture was maintained at 4^oC in MRS broth pH 5.5.

Identification of *Lactobacillus* species

Identification of the isolated bacteria as *Lactobacillus* species was performed according to their morphological, cultural, and physiological and biochemical characteristics by the procedures as described in Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994;Williams and Wilkins,1995). The tests carried out were Gram reaction, motility test, production of catalase, endospore test, milk coagulation activities, nitrate reduction test, urease test, H_2S production, starch hydrolysis test, sugar fermentation profile, production of ammonia

from arginine, 0.4% phenol tolerance test and NaCl tolerance (4%) assay.

Phenotypic characterization

1% (v/v) fresh overnight culture of the isolate was 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 overnight culture of the isolate.

Biochemical characterization

Ammonia production by arginine hydrolysis was performed in MRS broth containing 0.3% arginine and 0.2 % sodium citrate replacing ammonium citrate. Production of ammonia was detected by using Nessler's reagent.H_2S producing capacity was done by incubating the isolated culture in the medium containing casein peptone, meat peptone, sodium thiosulfate, Fe ammonium sulfate and agar for 2 weeks at 37^{o}C.The starch hydrolysis was performed on MRS agar medium with addition of 1% starch and incubation for 1 week at 37^{o}C.Nitrate reduction test was carried out by culturing the isolate in the medium containing peptone, potassium nitrate and sodium chloride.

Urease test was performed out by culturing the isolate in the medium containing peptone, sodium chloride, mono-potassium phosphate, glucose, urea, agar and phenol red.

For milk coagulation test 1% (v/v) fresh overnight culture of the isolate was inoculated into 10% sterilized skim milk and initial pH was recorded. The inoculated skim milk was incubated at 37^{o}C for 72 hr. After 72 hr liquids of coagulated milk were separated by filtration. pH of the separated liquid was recorded and organic acid produced were quantified through titration against 0.1 N NaOH.

The ability of the isolated culture to ferment different carbohydrates was examined on basal MRS broth (MRS devoid of glucose and meat extract and contain 0.004% chloramphenicol red indicator) with addition of different sugar solution to a final concentration of 2% (w/v). Acid production of the isolate by utilizing carbohydrates was evaluated at 24 hr.

Determination of optimum pH and temperature for growth

Optimum pH for growth of the isolate was determined by incubating 1% (v/v) fresh overnight culture into MRS broth at varying pH ranging from 3-9. For temperature assay, the culture was incubated at different temperature (15-50^{o}C) under anaerobic conditions. After 24 hr of incubation development of growth was measured by reading the optical densities at 560 nm against the uninoculated broth.

Assay for NaCland phenol tolerance

For the determination of NaCl tolerance, 1% (v/v) fresh overnight culture of the isolate was incubated into MRS broth adjusted with NaCl concentration of 4%. After 24 hr of incubation growth of the isolate was determined by observing their turbidity. Similar experiment was performed using 0.4% phenol used as inhibitory substance.

Screening of isolated *Lactobacillus* species for probiotic properties

Antibiotic susceptibility test

Disk diffusion method described by Andrews was followed to determine the sensitivity of the isolated culture to different antibiotics. Six different antibiotics such as

Penicillin, Rifampin, Isoniazid, Metrogyl, Amoxicillin and Tetracycline were collected and varying concentrations (0.25-1 mg/ml) of all the selected antibiotics were prepared in MRS broth. The test inoculum was prepared by incubating the isolated culture into MRS broth at 37^{o}C for 12 hr and 100 µl of it was inoculated to Muller-Hinton agar plates by spread plate method. 4 wells were made in each of the plates. These wells were filled with 100 µl of selected antibiotics each of different concentrations. Agar plates were then and incubated for 24 hr at 37^{o}C. The zone of inhibition was visualized.

Detection of antimicrobial activity

Well diffusion assay method was used for the detection of antimicrobial activity. The isolated culture was incubated for 48 hr in MRS broth at 37^{o}C. The cell free solution was obtained by centrifugation (10min x 10000 g) followed by filtration. 24 hr broth culture of target strains were inoculated on solid Muller-Hinton agar medium by spread plate method. 4 wells were made in each of the plates. These wells were filled with 100 µl of previously prepared cell free solution. Target strain inoculated plate with un-inoculated MRS broth served as control. The plates were incubated at 37^{o}C for 24 hr and the inhibitions zones were visualized.

Results and Discussion

Isolation and identification of *Lactobacillus* species

In the present study among all the bacteria isolated from local traditional dahi sample, only Gram positive and catalase negative isolates were chosen for further characterization. They were identified as *Lactobacilli* by observing their colony morphology, cultural, physiological and biochemical characterization.

Colony characteristics of *Lactobacilli* isolates were studied by picking-up a single well isolated colony aseptically and transferred to selective medium to observe the growth pattern of isolates on MRS medium. Colonies appeared creamy white colored, circular, low convex with entire margin were regarded as belonging to the genus *Lactobacillus*. Their distinguishing features are shown in (Table 1).

The *Lactobacillus* isolate exhibited negative pattern of H_2S formation, starch hydrolysis, nitrate reduction and urease activity. These are the common characters of *Lactobacillus* species. Similar observations were found by H. Forouhandeh *et al*, 2010 (Forouhandeh, 2010) in the isolation of *Lactobacillus* species from traditional cheeses and yogurts of Basmej Zone in Iran. The present experiment indicates that there was 27% organic acid production after 72 h incubation of skim milk with the isolated *Lactobacillus* species at 37^{o}C. Sugar fermentation patterns of isolated species (Table 1) indicated that fructose, glucose and lactose were fermented by isolated *Lactobacillus* species but maltose and gelatin were not fermented.

Determination of optimum pH and temperature for growth

From the experimental results (Table 2) it was observed that growth of the isolated *Lactobacillus* occurred within the pH range from 3-9 and the optimum pH value for good growth was at pH 5.5-6.5. This finding revealed that the isolated *Lactobacilli* species were able to survive in extreme acidic as well as alkaline conditions.

Similar results were observed by M.Z.Hoque, 2010 (Hoque *et al.*, 2010) in isolation, identification of *Lactobacillus*

species from regional yogurts in Bangladesh.

Like pH, temperature is an important factor in bacterial growth. In this study the isolated *Lactobacillus* species were able to grow within 30-50^{o}C and the optimum temperature for maximum growth was found at 37^{o}C.

Assay for NaCl and phenol tolerance

In the present study it was observed that (Table 2) *Lactobacillus* isolated from local dahi sample have the abilities to tolerate inhibitory substances such as 0.4% bacteriostatic phenol and good growth was also observed at 4% NaCl. The results have the similarities with findings of Eiezete and Carlos, 2005 (Elizete and Carlos, 2005).

Table.1 Morphological, physiological and biochemical characterization of the isolated bacterial strain

Morphological Tests

Colony Morphology	
Configuration	Round
Margin	Wavy
Elevation	Flat
Surface	Mucoid
Texture	Dry
Pigment	White-Creamy
Opacity	Opaque
Gram's reaction	+
Cell shape	Rod
Spore(s)	_
Motility	Non motile
Biochemical Tests	
H_2S formation	_
Catalase	+
0.4 % phenol tolerance test	+
5% NaCl tolerance test	+
Nitrate reduction	_
Arginine Dihydrolase	+
Urease	_
Gelatin hydrolysis	_
Starch hydrolysis	_
Organic acid production in skim milk	27%
Acid Production from	
Lactose	_
Maltose	_
Fructose	+
Glucose	+

'+' sign stands for positive and '-'stands for negative result

Table.2 Effect of medium pH, incubation temperature and NaCl on growth of the isolated *Lactoacillus* sp

Parameter	Medium pH							
	4.0	5.0	5.5	6.0	6.5	7.0	8.0	9.0
Growth	+	++	+++	+++	+++	++	+	-
	Incubation Temperature (^{o}C)							
	15	20	25	30	37	42	50	55
Growth	-	+	+	+	++	+	+	-
	NaCl (%)							
	2	3	4	5	6	7	8	9
Growth	++	+	+	+	+	+	+	-

'-' or'+' sign stands for no and positive visible growth andnumber of '+' sign stands for relative extent of visible growth

Table.3 Antibiotic sensitivity test

Antibiotics	Dilution	Sensitivity
Amoxicillin	0.25-1.0 mg/ml	+
Tetracycline	0.25-1.0 mg/ml	+
Penicillin	0.25-1.0 mg/ml	+
Rifampicin	0.25-1.0 mg/ml	+
Metrogyl	0.25-1.0 mg/ml	+

'+' sign stands for visible growth

Table.4 Antibacterial activity of the isolated bacterial strain

Test Organisms	Gram Character	Growth of test organisms
Escherichia coli	Negative	Inhibited
Bacillus subtilis	Negative	Inhibited
Staphylococcus aureus	Positive	Inhibited
Pseudomonas spp.	Negative	Inhibited

Screening of isolated *Lactobacillus* species for probiotic properties

Antibiotic susceptibility test

Results concerning the sensitivity of the isolated *Lactobacillus* species to different antibiotics are shown in Table 3 which reveals that isolates were resistant to all the five selected antibiotics including amoxicillin indicating that antibiotics will not affect the growth of the isolated *Lactobacillus* population.

Detection of antimicrobial activity

Table 4 represents the antimicrobial activities exhibited by *Lactobacillus* species

which indicates that the cell free solution of isolated *Lactobacillus* species were able to inhibit the growth of all the test microorganisms. This experiment clearly indicates that the inhibitory metabolites produced by isolated *Lactobacillus* species were extracellular and diffusible. These results are in accordance with those reported by Arpita *et al* (Patra *et al.*,2011).

The experimental results showed that the traditional fermented milk product dahi contain *Lactobacilli* which can tolerate inhibitory substances and were able to survive both in acidic and alkaline conditions. They exhibited antimicrobial activity against some indicator pathogens and were resistant to different antibiotics. Based on these characteristics the isolates may have potential for natural preservatives and may also be considered for probiotic application.

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