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Research Article

ISOLATION, CHARACTERISATION AND IDENTIFICATION OF LACTOBACILLI SPP. AND STUDY OF ITS PHARMACOLOGICAL ACTIVITY IN VITRO

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ABSTRACT

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* specie are resistant to inhibitory substances like phenol (0.4%) and NaCl (4%). 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

Probiotics are live microorganisms used as food supplements, which provide health benefits when consumed, through improving the intestinal microbial balance of the host. They are live microorganisms which when administered in adequate amounts confer health benefits on the host. Microorganisms commonly used as probiotics include *Bifidobacteria, Lactobacilli* and certain yeasts. Research has shown that addition of probiotics to food provides many a health benefit. They are able to increase the frequency of bowel movements and stimulate cell-mediated immunity[1].The probiotics have been suggested as way to step into a more environment friendly aquaculture by reducing the use of chemicals and antibiotics [2,3]. *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 [4].

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 the isolation, characterisation and identification of Lactobacilli spp. and study of its pharmacological activity in vitro.

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 Panskura, 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*. Three different antibiotics were purchased from local medicinal shop.

Bacterial strain and culture conditions

Two Gram negative and two Gram positive bacteria used for antimicrobial assay respectively, *Escherichia coli* (MTCC 443), *Klebsiella pneumonia* (MTCC 109), *Staphylococcus aureus* (MTCC 3160) and *Salmonela typhi* (MTCC 890) were provided by National Cholera Institute, Kolkata. 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.

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Isolation of Lactobacillus

Lactobacillus was isolated from locally available dahi by using de Mann Rogosa Sharpe (MRS) agar media[5].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^{o}C 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^{o}C 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[6]. The tests carried out were Gram reaction, motility test, production of catalase, Indole, Methyl Red, Voges-Proskauer, Citrate, Starch Hydrolysis, endospore test, milk coagulation activities and NaCl and phenol tolerance test.

Assay for NaCl and 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 [7].

Assay of milk coagulation test

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 37oC for 72 hr. After 72 hr liquids of coagulated milk were separated by filtration[7].

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. Three different antibiotics such as Azithromysin, Ampicillin, 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 uninoculated 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 colour, circular, low convex with entire margin were regarded as belonging to the genus *Lactobacillus*. Their distinguishing features are shown in (Table 1 and Figure 1). Their biochemical characters are also shown in the (Table 2).

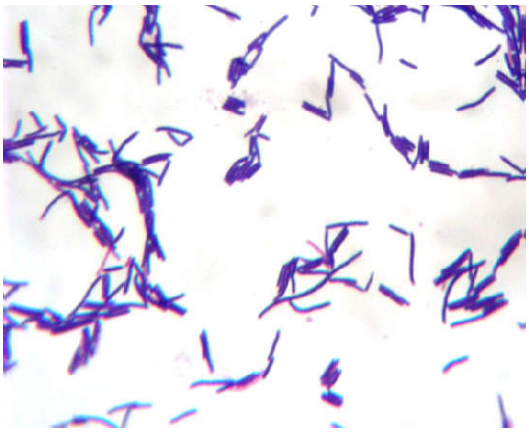

Figure 1 The isolated rod shaped bacteria by phase contrast microscopy.

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

Colony Morphology	
Configuration	Round
Margin	Wavy
Elevation	Flat
Surface	Mucoid
Texture	Dry
Gram's Reaction	+Ve
Cell Shape	Rod
Spore(s)	-Ve
Motility	Motile

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.

Table.2 Biochemical characterization of the isolated bacterial strain.

Biochemical Tests	
Catalase	-Ve
Indole	-Ve
Methyl Red	-Ve
Voges-Proskauer	-Ve
Citrate	-Ve
Starch Hydrolysis	-Ve
0.4 % phenol tolerance test	+ve
5% NaCl tolerance test	+ve

+ sign stands for positive and - stands for negative result

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 three selected antibiotics indicating that antibiotics will not affect the growth of the isolated *Lactobacillus* population.

Table 3 Antibiotic sensitivity test

Antibiotics	Dilution	Sensitivity
Azithromysin	0.25-1.0mg/ml	+
Ampicillin	0.25-1.0mg/ml	+
Tetracycline	0.25-1.0mg/ml	+

Detection of antimicrobial activity

Table 4 represents the antimicrobial activities exhibited by *Lactobacillus* specieswhich 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 [8] and shown in the (Table4).

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.

Table 4 Antibacterial activity of isolated bacterial strain

Test organisms	Gram character	Growth of test organisms
Escherichia coli (MTCC 443)	Negative	Inhibited
Klebsiella pneumonia(MTCC 109)	Negative	Inhibited
Staphylococcus aureus (MTCC 3160)	Positive	Inhibited
Salmonela typhi(MTCC 890)	Positive	Inhibited

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