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RESEARCH PAPER

Isolation and screening of lactic acid bacterial isolates from sorghum and *Ragi* grains for its probiotic characters

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ABSTRACT

Twenty one isolates were obtained from two different sources like fermented Sorghum and *Ragi* grains. Out of which eleven isolates obtained from Sorghum grains. Remaining ten isolates were from *Ragi* grains. Microscopic observation and morphological characterization of the all twenty one isolates confirmed that they were Gram positive, mostly short rods and coccobacilli. The physiological and biochemical characterization confirmed that the isolates used were lactobacilli. All these isolates were screened for its probiotic characters such as antimicrobial activity against food spoilage organisms and also for bile tolerance and pH tolerance.

Key Words : Lactic acid bacteria, Probiotic

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The term (LAB) Lactic acid bacteria denote a rather diverse group of bacteria. They have been used to ferment or culture foods for at least 4000 years. They commonly occur in milk products, fermented products and observed on the surfaces of fruits and vegetables. LAB includes the main genera *Lactobacillus* and *Bifidobacterium*, which has the lowest risk to humans and beneficial to us (Sanni *et al.*, 1999). Lactic acid bacteria have shown to play an important physiological role in the human gastrointestinal (GI) tract. Microbial interactions represent the main force in the homeostasis of the bacterial flora in the GI tract. The importance of maintaining a beneficial microbial balance in the GI tract is vital to the stability of its ecosystem and the optimal health of the host. The bacteria in the LAB genera are classified by their cell morphology and the fermentation pathway used to ferment glucose. Probiotics are live micro-organisms, administered in quantities adequate to confer health benefits (FAO, 2007). LAB strains isolated from fermented foods have been used as probiotics due to their resistance to host gastrointestinal conditions, adhesion to host intestinal epithelium and the prevention of the growth or invasion of pathogenic bacteria into the animal intestine (Chiu *et al.*, 2007). The most important LAB is *Leuconostoc*, *Lactobacillus*, *Streptocccus* and *Pediococcus*. *Lactobacillus acidophilus* has been considered the predominant *lactobacilli* in the intestinal tract of healthy

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humans and, therefore, is the organism most commonly used in probiotic products.

RESEARCH METHODOLOGY

Sample collection :

The grain sample of the *Ragi* (*Eleusine coracana*) variety CO 14 and sorghum variety was collected from Millet Breeding Station, Tamil Nadu Agricultural University, Coimbatore for this study.

Fermentation of substrates :

A quantity of 100 g of sorghum and *Ragi* grains were soaked separately in 150 ml of sterilized drinking water. It was allowed to ferment naturally for 24 h at room temperature (30 °C). The soaked grains were ground to slurry using pestle and mortar. At time interval of 24 h of incubation, the samples of fermented soaked grains and fermented slurry were taken for analysis.

Isolation and Purification of lactic acid bacteria :

Lactic acid bacteria (LAB) were isolated from the soaked grains and fermented slurry by adequately diluting the samples and plating on Man Rogosa Sharpe (MRS) agar medium (De Man *et al*., 1960). The plates were incubated at 37°C in an incubator (New Brunswick, USA) for 48 h. After the period of incubation the plates were observed for colony growth and morphological characters. Single colonies from the purification plate were further inoculated on MRS agar plate. Selected isolates were purified on MRS agar medium by streak plate method and allowed to grow at 34°C for 24 h. The single colony was transferred to MRS agar slants and incubated at room temperatures. After sufficient growth, the slants were stored in refrigerator at 4°C for further investigation.

Morphological characterization of LAB isolates :

Gram staining :

The Gram's reaction of the isolates was done by staining, to identify the morphological structure of lactic acid bacterial isolates as per method of Claus (1992). All the isolates were characterized by Gram Staining.

Cell shape and size :

All the isolates were simple stained with crystal violet and observed under 45X of compound microscope to view the cell shape and the size was measured by micrometry using an ocular micrometer calibrated with a stage micrometer (Erma, Japan).

Biochemical characterization :

The LAB isolates were identified biochemically using various tests. These include the following biochemical tests as described by Aneja, 1996 *viz.*, catalase test, Methyl Red - Voges Proskauer test, Citrate Utilization test, Nitrate reduction test, Nitrite reduction, Litmus milk reaction, starch hydrolysis, sugar fermentations utilization.

Utilization of various carbon sources by LAB isolates:

The isolates of LAB were inoculated into fermentation broth with different carbon sources *viz.*, glucose, fructose, sucrose, lactose, raffinose, sorbitol, salicin, maltose, galactose and mannitol. Durham tube was inserted in an inverted position and kept for 48 h of incubation at 37 °C. Change in the colour of the fermentation broth and appearance of gas bubbles were observed by comparing with an uninoculated broth.

Screening of lactic acid bacterial isolates for antimicrobial activity :

The lactic acid bacterial isolates were primarily screened for antimicrobial activity using food borne pathogenic micro-organisms like *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*. The strains of *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* were obtained from the culture collection of the Department of Microbiology, Coimbatore. Micro-well diffusion agar assay is used for detection of antimicrobial activity (Schillinger and Lucke, 1987). Agar plates seeded with cultures of indicator organisms were prepared. In the plate, at the centre a well of diameter 5 mm was cut and 50 µl of the test LAB isolate was placed into the well. Well loaded with sterile distilled water served as control. The MTCC 10307 *Lactobacillus acidophilus* culture was also included for comparison. The probiotic strain plates were incubated at 37 °C for 24 to 48 h and observed for inhibition zone around well.

Screening of acid tolerant isolates :

Each isolate was grown in MRS broth for 24 h. After this incubation, about 100 µl of the inoculum (1%) was inoculated into MRS broth acidified with concentrated hydrochloric acid (1 N) to pH 5.0, 4.0 and

3.0. A non-acidified control (pH 6.0) was also maintained. Three replications were maintained for each isolate. The tubes were incubated for 48 h while the absorbance at 610 nm (A_{610}) was monitored at 2h intervals. By plotting time interval in 'X'-axis and absorbance in 'Y'-axis, the growth curve was obtained. The log phase time for the culture was determined and it was used for further investigations in the study. Plate counts from each treatment were taken using MRS agar (pH 6.0) by following pour plate technique at 48 h. On addition of acid, isolates that showed good growth were considered acid tolerant.

RESULTS AND REMONSTRATION

The results obtained from the present investigation as well as relevant discussion have been summarized under following heads :

Isolation and purification of lactic acid bacteria :

The lactic acid bacterial isolates were obtained from soaked water and fermented slurry of Sorghum variety CO 2 and *Ragi* variety CO 14 kept for overnight by pour plate method and purified by streak plate method on MRS medium. The pH of fermented slurry and soaked water of grains decreased from 7.6 to 5.4 and 7.1 to 5.8 over the incubation time respectively. Of the total number of twenty one isolates, 11 isolates were obtained from Sorghum grains, out of which 7 isolates from fermented slurry and 4 isolates from soaked water and remaining 10 isolates were obtained from *Ragi*, out of which 6 isolates from fermented slurry and 4 isolates from soaked water respectively. The LAB isolates obtained from Sorghum were designated and numbered as LAB 1 to LAB 11 and from *Ragi* were designated and numbered as LAB 12 to LAB 21.

Characterization of LAB isolates obtained from fermented millets :

Morphological characterization :

The morphological characteristics of all the twenty one LAB isolates were studied by the microscopic observation and the details are presented in Table 1. The colony characteristics of the isolates grown in MRS agar plates were recorded based on the colour, elevation and form. The colony of the isolates that appeared on MRS

Table 1 : Morphological characteristics of the lactic acid bacterial isolates obtained from sorghum and *Ragi* grains

Isolates	Colony morphology	Cell morphology	Gram's reaction
LAB 1	Creamy white, small, round, raised	Long rods in chains of two	+
LAB 2	Creamy white, small, irregular, flat	Coccobacilli in singles	+
LAB 3	Milky white, big, round, raised	Long rods	+
LAB 4	Creamy white, fairly big, irregular, flat	Long rods	+
LAB 5	Dull white, big, round, flat	Short rods in chains of two	+
LAB 6	Milky white, big, round, raised	Long rods	+
LAB 7	Creamy white, fairly big, round, flat	Short rods in chains of two	+
LAB 8	Dull white, fairly big, round, raised	Coccobacilli in singles	+
LAB 9	Creamy white, big, irregular raised	Coccobacilli in chains	+
LAB 10	Dull white, fairly big, round, raised	Short rods	+
LAB 11	Creamy white, small, round, raised	Coccobacilli in singles	+
LAB 12	Creamy white, small, irregular, flat	Long rods in chains of two	+
LAB 13	Milky white, big, round, raised	Short rods	+
LAB 14	Creamy white, fairly big, irregular, flat	Long rods	+
LAB 15	Dull white, big, round, flat	Coccobacilli in chains	+
LAB 16	Milky white, big, round, raised	Short rods	+
LAB 17	Creamy white, fairly big, round, flat	Long rods	+
LAB 18	Dull white, fairly big, round, raised	Short rods in chains of two	+
LAB 19	Creamy white, big, irregular raised	Long rods	+
LAB 20	Dull white, fairly big, round, raised	Short rods in chains of two	+
LAB 21	Creamy white, small, round, raised	Coccobacilli in singles	+
*MTCC 10307	Small, brownish, entire	Long rods in chains of three to four	+

* Standard culture –*Lactobacillus acidophilus*

LAB 1 to LAB 11 –Lactic acid bacteria from Sorghum LAB12 to 21 - Lactic acid bacteria from Ragi

plates after incubation were small, medium and big sized and non pigmented (dull cream coloured and white). Most of them were raised, while others were flat.

Smears of all isolates, prepared and simple stained with crystal violet, were observed under 45 x of compound microscope, which indicated that ten of the isolates were long rods, seven were short rods and about four were coccobacilli. All the isolates stained were found gram positive. Based on study of gram's reaction, morphological characterization and biochemical characterization, it was confirmed that isolates are *Lactobacilli*. Most of the colonies of LAB isolates used in this study appeared big and cream coloured. According to Kandler and Weiss(1986) members of the genus *Lactobacillus* are gram positive short - long rods in chains. Tharmaraj and Shah (2003) also reported that the colony of *L. delbrueckii* sp *bulgaricus* were 1.0 mm in size, white, cottony, rough and irregular on MRS agar (pH 4.58) when grown under anaerobic conditions at 37°C for 72 h.

Biochemical tests :

In order to tentatively identify the isolates, various biochemical tests like catalase test, MR-VP test, citrate utilization test, nitrate reduction, nitrite reduction, starch hydrolysis, litmus milk test and carbohydrate fermentation test were carried out. All the isolates were tentatively identified as *Lactobacillus*. The results of biochemical tests are presented in Table 2 and 3.

In the present study, all the isolates showed absence of catalase activity which readily demonstrated by the presence of O_2 formation is one of the most useful diagnostic tests for the recognition of these organisms, since they are virtually the only bacteria devoid of catalase that can grow in the presence of air.

All the isolates used in this study showed colour change during litmus milk reaction. This indicated that the milk sugar lactose has been fermented by the production of acid. When tested for starch hydrolysis, all the isolates were found to have starch hydrolysing enzymes confirmed by the presence of clearing zones beneath and around the cultures. Some species of *Lactobacillus* like *L.amylophilus* are found to hydrolyse starch.

All the isolates have been found to utilize lactose, glucose, sucrose and galactose. Most of the LAB isolates

Table 2 : Biochemical characterization of the lactic acid bacterial isolates from fermented millets

Isolates	Catalase test	Methyl red test	Voges-proskauer test	Citrate utilization test	Nitrate reduction test	Nitrite reduction test	Litmus milk test	Starch hydrolysis test
LAB 1	-	+	-	+	+	-	+	+
LAB 2	-	-	-	+	-	-	+	+
LAB 3	-	+	-	+	+	-	+	+
LAB 4	-	-	-	+	+	-	+	+
LAB 5	-	-	+	-	-	-	+	+
LAB 6	-	-	+	+	+	-	+	+
LAB 7	-	+	-	+	-	+	+	+
LAB 8	-	+	-	-	+	-	+	+
LAB 9	-	+	-	+	+	-	+	+
LAB 10	-	+	-	+	+	-	+	+
LAB 11	-	+	+	+	-	-	+	+
LAB 12	-	-	-	-	+	-	+	+
LAB 13	-	-	-	+	-	+	+	+
LAB 14	-	-	-	-	+	-	+	+
LAB 15	-	+	-	-	+	-	+	+
LAB 16	-	+	-	+	-	+	+	+
LAB 17	-	+	-	+	-	-	+	+
LAB 18	-	+	-	-	+	-	+	+
LAB 19	-	-	-	+	+	-	+	+
LAB 20	-	-	+	+	+	-	+	+
LAB 21	-	-	-	-	-	-	+	+
MTCC 10307	-	+	-	+	-	-	+	+

+ = Positive, - = Negative

found to utilize mannitol, Similar results were obtained by Tharmaraj and Shah (2003) who found that two strains of *Lactobacillus acidophilus* (LA-LAC 4 and LA-74-2) grew well in basal media containing the sugar mannitol at 37°C.

In the present study, only 4 isolates LAB 3,7,13 and 20 did not utilize mannitol, remaining 17 isolates have utilized mannitol, and among 21 isolates, LAB 4,12,16 and 20 did not utilize salicin. Shah (2000) also observed that 6 strains of *Lactobacillus acidophilus* utilized sugars like salicin, cellobiose, fructose, mannitol and glucose. In the same study, *L. delbrueckii* ssp. *bulgaricus* was found to utilize only fructose and glucose. Certain strains of *Lactobacillus* were found to use mannitol as their carbon source. Some examples *are L. casei, L. acetotolerans, L. agilis, L. coryniformis, L. coryniformis* ssp *torquens, L. paracasei* sp. *paracasei, L. pentosus, L. rhamnosus, L. plantarum* and *L. sake*. Based on sugar fermentation patterns they concluded that salicin was suitable for selective enumeration of *L. acidophilus*. In a study on the fermentation of sugars, Kaplan and Hutkins (2000) indicated that many strains of *L. acidophilus* including the strains DDS-1 and NCFM fermented fructooligosaccharides, which consists of a glucose monomer linked to two or more fructosyl units. It was also found that *L. casei* and *L. plantarum* utilized FOS. *Lactobacillus bulgaricus* fermented, lactose, glucose, fructose and mannose and did not grow on galactose (Grobben *et al.*, 1998).

Screening of LAB isolates for antagonistic activity:

Antimicrobial activity is an important feature for selection LAB for designing starter cultures for industry. All the LAB isolates were screened based on antagonistic activity against food spoilage organisms (test organisms) *viz., Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus* using agar well diffusion assay. The results for antagonistic activity of the LAB isolates which was measured in terms of inhibition zone (Diameter in mm) formed are presented in Table 4.

Of all the isolates screened for antagonistic activity, all isolates in general has highest antagonistic activity against *Bacillus subtilis,* when compared to *Escherichia coli and Staphylococcus aureus*. The isolates LAB 4, 5, 8, 9, 10, 13, 14, 15, 17, 18 and 20 had no inhibitory

Table 3 : Utilization of carbon sources by lactic acid bacterial isolates

Isolates	Glucose	Fructose	Sucrose	Galactose	Maltose	Lactose	Salicin	Sorbitol	Mannitol	Raffinose
LAB 1	+	-	+	+	+	+	+	+	+	+
LAB 2	+	+	+	+	-	+	+	+	+	-
LAB 3	+	+	+	+	+	+	+	-	-	-
LAB 4	+	+	+	+	-	+	-	+	+	-
LAB 5	+	+	+	+	+	+	+	+	+	-
LAB 6	+	+	+	+	+	+	+	+	+	+
LAB 7	+	+	+	+	+	+	+	-	-	-
LAB 8	+	-	+	+	+	+	+	+	+	+
LAB 9	+	-	+	+	+	+	+	+	+	-
LAB 10	+	+	+	+	+	+	+	+	+	-
LAB 11	+	+	+	+	+	+	+	+	+	+
LAB 12	+	-	+	+	-	+	-	-	+	+
LAB 13	+	+	+	+	+	+	+	+	-	-
LAB 14	+	-	+	+	+	+	+	+	+	-
LAB 15	+	-	+	+	-	+	+	+	+	-
LAB 16	+	+	+	+	+	+	-	+	+	+
LAB 17	+	-	+	+	+	+	+	+	+	-
LAB 18	+	-	+	+	+	+	+	+	+	-
LAB 19	+	+	+	+	-	+	+	-	+	+
LAB 20	+	+	+	+	+	+	-	+	-	+
LAB 21	+	-	+	+	+	+	+	+	+	-
MTCC 10307	+	+	+	+	+	+	+	-	-	-

+ = Positive - = Negative

activity against *Escherichia coli.* LAB 5, 10, 17, 18 and 19 have exhibited inhibitory activity over *Staphylococcus aureus*. Among the twenty one isolates, LAB 7 had shown highest antagonistic activity over test organisms *viz., Bacillus subtilis* (12.6 mm), *Staphylococcus aureus* (7.0 mm) and *Escherichia coli* (6.5 mm) followed by LAB 12 over *Bacillus subtilis* (11.5 mm), *Staphylococcus aureus* (6.3 mm) and *Escherichia coli* (6.1 mm), when compared to other isolates. Hence, LAB 7 isolate was selected as efficient strain with highest antagonistic activity. LAB form many antibacterial substances (organic acids, carbon dioxide, ethanol, hydrogen peroxide, diacetyl, antifungal compounds, bacteriocins, antibiotics, fatty acid, phenyllactic acid), which have positive influence on shelf-life of fermented product (Valerio *et al.*, 2008).

At present, Nisin is the only bacteriocin active against gram positive bacteria, commercially available and marketed (Balasubramanyam and Varadaraja, 1998). Nisin has an inhibitory effect against a wide variety of gram positive food borne pathogens and spoilage micro-organisms (Rodriguez, 1996) and can also act on several gram negative bacteria when the integrity of their outer membrane is disrupted. Of the different LAB isolates LAB 7 and LAB 12 isolates showed largest spectrum of antibacterial activity against certain gram negative bacteria containing *Escherichia coli* and also gram positive *Bacillus subtilis* and *Staphylococcus aureus*. The antimicrobial compound produced by the test organisms has some interesting characteristics that justify this study. The survival of proliferation of an organism can be envisaged, if it can eliminate competing organisms by inhibitory compounds, lytic agents, lytic enzymes or metabolic by-products like organic acids, hydrogen peroxide and diacetyl. These results indicated that the LAB isolates are capable of synthesizing inhibitive substances produced by LAB strains act differently on the pathogenic reference indicator strains. The antimicrobial effect is believed to result from the action of the acids in the bacterial cytoplasmatic membrane, which interferes with the maintenance of the membrane potential and inhibits the active transport. Apart from their ability to produce organic acids, the LAB posses the ability to produce hydrogen peroxide through the

Table 4 : Antibacterial activity of lactic acid bacterial isolates from fermented millets

Isolate number	Target organisms/ Diameter of inhibition zone (mm)		
	Escherichia coli	*Bacillus subtilis*	*Staphylococcus aureus*
LAB 1	2.1	3.6	4.3
LAB 2	0.9	4.7	1.2
LAB 3	5.4	11.2	4.4
LAB 4	-	3.6	2.1
LAB 5	-	2.1	-
LAB 6	5.6	11.0	5.4
LAB 7	6.5	12.6	7.0
LAB 8	-	4.8	1.2
LAB 9	-	4.7	1.3
LAB 10	-	3.2	-
LAB 11	3.5	9.8	4.5
LAB 12	6.1	11.5	6.3
LAB 13	-	2.8	5.6
LAB 14	-	4.2	4.7
LAB 15	-	4.5	3.0
LAB 16	3.7	10.2	5.1
LAB 17	-	11.1	-
LAB 18	-	6.9	-
LAB 19	4.3	10.0	-
LAB 20	-	5.8	4.4
LAB 21	5.4	3.3	1.3
MTCC 10307	2.5	11.0	6.0

Values represent mean of three replications

oxidation of reduced nicotin-amide adenine dinucleotide (NADH) by flavin nucleotides, which react rapidly with oxygen. As LAB lack true catalase to break down the hydrogen peroxide generated, it can accumulate and be inhibitory to some micro-organisms (Caplice and Fitzgerald, 1999).

Screening of LAB isolates based on their pH tolerance:

All the isolates were inoculated into MRS broth acidified with hydrochloric acid (1N) to adjust pH of the medium to 3.0, 4.0 and 5.0 for testing the pH tolerance. The results are presented in the Fig. 1, 2 and 3.

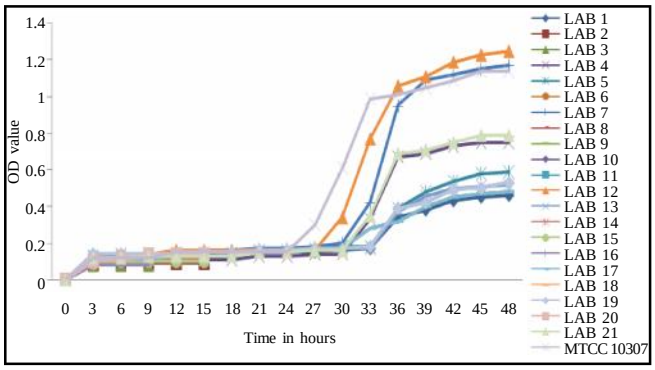

Fig. 1 : Growth of LAB isolates in MRS broth at pH 3.0

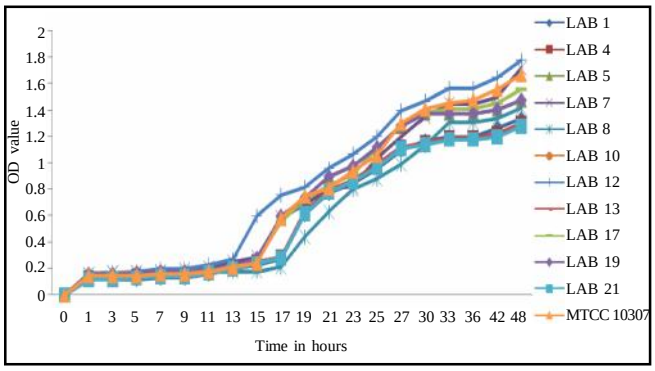

Fig. 2 : Growth of LAB isolates in MRS broth at pH 4.0

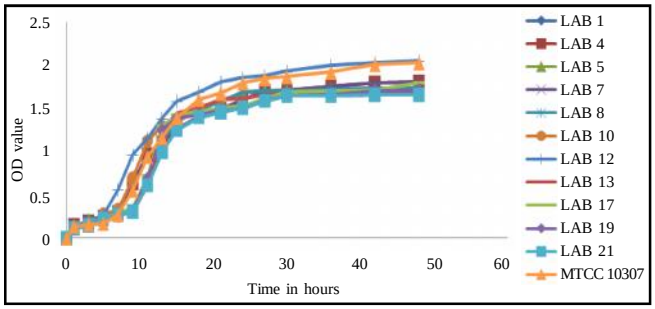

Fig. 3 : Growth of LAB isolates in MRS broth at pH 5.0

Optical density values recorded at 610 nm (A_{610}) at 3h intervals upto 48 h shown that all the isolates were not tolerant to pH 3.0. Among all the 21 isolates, only 11 isolates *viz.*, LAB 1, 4, 5, 7, 8, 10.12,13,17,19 and 21 have shown tolerance to pH 3.0. The remaining isolates LAB 2,3 6, 9, 11,14,15,16,18 and 20 did not show any growth at pH 3.0. Poor survival of some isolates may be attributed to the genetic makeup of the strain (*L. acidophilus* usually tolerates low pH while others do not) or may be due to lysing of cells at these adverse conditions when exposed for long periods.

Among all the isolates, LAB 12 and 7 shown high OD values and population counts at the pH 3.0 when compared to other isolates.

All the isolates that are tolerant to pH 3.0 also showed good growth at pH 4.0 and pH 5.0 The time taken to reach log phase decreased in pH 5.0, when compared to pH 4.0 and pH 3.0. LAB 12 shown maximum OD value (2.06) with population reaching 10^9cfu.ml^{-1} at the pH 5 after 48 h. The LABC 6 isolate had shown best tolerant level at all the three different pH 3.0, 4.0 and 5.0 tested, when compared to all the other isolates and over the reference culture MTCC 10307 (*Lactobacillus acidophilus*). Hence, LAB 12 isolate was selected as efficient acid tolerant.

Among the 21 LAB isolates used in the present study, only LAB 12 was found to have high counts upto 10^7 cfu / ml at all acidifying conditions. At pH 3.0 LAB 12 and LAB 7 showed maximum population 7.52 log cfu/g and 6.35 log cfu/g at 48 h, respectively. This is similar to the findings of Chou and Weimer (1999) who reported viable counts of *L. acidophilus* strains after 90 minutes of incubation at 37°C to be 8.1- 9.2 log. Similar reports were made by Marteau *et al.* (1997) who concluded that more than 40 per cent of the ingested strain of *L. acidophilus* ($3.6x10^6$) remained viable at 120 min of incubation in a dynamic model of intestine (at < pH 3.0). They also noted that viable counts of *L. bulgaricus* fell below 1 per cent of the ingested numbers of bacteria. Vinderola *et al.* (2000) studied the cell viability of *Lactobacillus* sp and *Bifidobacterium* at low pH levels (2 and 3) during 30, 60, 120 and 180 minutes after incubation who also found that *Lactobacillus lactis* decreased 3 log orders after 3 h in both acidic conditions. At pH 3.0 *L. casei* and *L. acidophilus* was reduced by only 1.3 log orders. At pH 2.0, the probiotic starter bacteria and *L. acidophilus* were not able to survive for 1 h, while counts of *B. bifidum* and *L.casei* lowered

by 5 and 7 log orders, respectively.

A study conducted by Chang *et al*. (2001) revealed that a strain identified as *L. reuteri* BSA 131 had tolerance (growth in oxgall or survival in pH treated for 3h) at pH 2.0 and pH 3.0. Therefore, pH tolerance of lactobacilli differ from strain to strain (Mitsuoka, 1984). In another study, for screening of probiotic activities of different strains of *Lactobacillus* sp. Jacobsen *et al*. (1999) agreed that they observed survival of 29 strains at pH 2.5 following 4 h of incubation, but none seemed to replicate.

Conclusion :

Lactic acid bacteria are gaining momentum in the biopreservation technology. LAB was widely used in the fermentation processes. Foods that contain lactic acid bacteria and are able to shorten, alleviate or even prevent diarrhea need to be developed in the developing world In the food industry, LAB is widely used as starters to achieve favourable changes in texture, flavour, etc. In recent years, there has been increasing interest in using bacteriocin and or other inhibitory substance producing LAB for non-fermentative biopreservation applications. The probiotic property of the native isolates can be used for developing functional foods so can be used in food industry.

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