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Isolation and Screening of Probiotic Potential Lactic Acid Bacteria from Local Dairy Products

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A B S T R A C T

The aim of this study was to isolate and identify Lactic acid bacteria having probiotics property from Curd and buttermilk. There were 77 different bacteria isolated from curd and buttermilk. Among 77 isolates, potential bacterial isolates SS-1, SS-2 and SS-3 were further selected to evaluate their probiotic properties. The selected bacterial isolates were found to resistant at low pH 3 and with 0.5% bile salts concentrations. The isolates SS-1, SS-2 and SS-3 showed significant antibacterial activity against *E. coli* (MTCC-1610), *S. aureus* (MTCC-1430), *B. subtilis* (MTCC-441), and *P. vulgaris* (MTCC-1771). Present study indicated that local dairy products can be used as a good source for isolation and screening of novel potential probiotic isolates.

Introduction

Isolation and screening of microorganisms from naturally occurring processes have always been the most powerful means for obtaining useful cultures for scientific and commercial purposes. This is certainly true for lactic acid bacteria (LAB), which play major attention for their widespread use in the production of fermented foods (Farnworth, 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 (Coeuret *et al.*, 2003) and have tremendous industrial application (Stiles, 1996). The art of lactic acid fermentation is one of the oldest technologies that preserve the milk in a wide diversity of flavor, aromas and texture (Minamiyama *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, nonspore 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 nonpathogenic and do not produce toxic substances hence designed as "Generally Regarded as Safe" (GRAS). In recent years much attention is being given to isolation of *Lactobacilli* 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. The role of fermented milk in human diet was known from Vedic times. About 9% of the total milk is converted into fermented dairy product in India. Curd (Dahi), Buttermilk (chhass), sweetened yoghurt (lassi) are some of the traditionally fermented dairy products being used in every household of the Indian subcontinent. Among them Curd stands next to whole milk especially during summer. It is very easy to digest and enriched with vitamins, amino acids, pre digested protein and bioactive peptides, etc. (Campbell-Platt G., 1994).

In the Ayurveda Curd has been recommended for treatment of diarrhea and other acute/chronic gastrointestinal disorders from time immemorial. Lactic acid bacteria are the dominant microorganism found in Curd (Rashid *et al*., 2007) Curd 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 dairy products. In these products, the species combination of LAB is more varying and inconsistent as compared to those of the trade products. The dairy industries require well-defined single strain and multiple strain starter cultures to obtain dairy products of high and constant quality. Therefore, a continuous need exists for the isolation of new strains with superior natural qualities. To provide health benefits by *Lactobacilli* present in dairy products, they require their relevant characterization and identification. The present study has been carried out with an objective to screen potential probiotic important novel species of *Lactobacilli* from fermented milk products from local dairy products vendors at Himatnagar, Dist. Sabarkantha, Gujarat and to study probiotic properties of isolated LAB.

Materials and Methods

Collection of Samples

Curd and buttermilk samples were collected from Local dairy products vendors of Himatnagar, Dist. Sabarkantha, Gujarat. Immediately after collection, the samples were stored aseptically in low temperature (4°C) refrigerator to protect from contamination and spoilage.

Isolation of Lactic Acid Bacteria

Approximately 10.0 g of each Curd or buttermilk samples were mixed with 90 ml of sterile peptone water (0.1 % w/v in distilled water), homogenized gently, diluted appropriately and spread plated aseptically on de Mann Rogosa Sharpe (MRS) agar media. Duplicate plates were incubated at 37°C for 48 hours in aerobic condition. Plates having less than 30 colonies were selected. Colonies differ in morphology, pigmentation, shape, and size were sub cultured to obtain purity in MRS broth. Purification of the isolates was confirmed by using various biochemical tests as described by Harrigan, 1998; Aneja, 2006). Samples were then purified by streaking and were maintained on MRS slant at 4°C.

Physiological and biochemical tests

All strains were initially tested for Gram reaction and catalase production activity

(Harrigan, 1998; Aneja, 2006). Only Gram positive and catalase negative were further identified.

Gas production from glucose

In order to determine the homofermentative and heterofermentative characterization of isolates, CO_2 production from glucose test was applied. Sugar tube with inverted Durham tubes were prepared and inoculated with 1% overnight fresh cultures. Then test tubes were incubated at 37 °C for 24 h. After 24 h observed gas occurrence in Durham tubes for CO_2 production from glucose.

Sugar fermentation

Three SS-1, SS-2 and SS-3 isolates were selected and subjected to sugar fermentation test. Fermentation of different sugars was studied in order to observe the growth of culture in Nutrient broth containing with 1% glucose, lactose, maltose, Fructose and mannitol individually.

Ability of growth at different temperature and salt concentration

Tolerance to different temperature and salt concentration was determined by growing the isolate in MRS broth at different temperature 10°C, 15°C, 37°C and 45°C and for salt tolerance MRS media supplemented with 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8% and 9% of sodium chloride concentration. After 24h, the test cultures were observed for growth, indicated by turbidity in the medium.

Probiotic properties of Isolates

For the determination of probiotic properties of isolates these following major selection criteria were selected:

Resistance to Low pH

Being resistance to low pH is one of the major selection criteria for probiotics strain (Quwehand *et al*., 1999). Since, to reach the small intestine they have to pass through from the stressful conditions of stomach (Chou and Weimer, 1999). For acid tolerance selected strains were inoculated into MRS broth varying concentration of pH (2, 3, 4, 5, 6 and 8) and OD at 620 nm was taken after 24 h incubation at 37°C against the control (pH 7.0).

Tolerance against bile

The Strains, resistant to low pH, were screened for their ability to tolerate the bile salt. Although the bile concentration of the human gastro intestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v and the staying time is suggested to be 4h (Prasad *et al*., 1998). For bile tolerance of selected strains were inoculated into MRS broth varying concentration of bile salt(0.05, 0.10, 0.15, 0.30 and 0.50%) and also MRS broth without bile salt as control and incubated at 37°C for 24 h. Optical density (OD) of each culture was recorded at 620 nm after incubation.

Antimicrobial activity

The selected strains were examined according to their antimicrobial activity. For this purpose, strains were detected against the indicator organisms *Escherichia coli (*MTCC-1610), *S.aureus* (MTCC-1430), *B.subtilis (*MTCC-441) and *P.vulgaris* (MTCC-1771)

Antibiotic sensitivity testing

The antibiotic resistance of Lactobacillus species was assessed using different

antibiotic discs on MHA plate seeded with the isolated strains. The antibiotics discs were placed on the surface of agar and the plates were kept at 4 °C for 1h for diffusion and then incubated at 37 °C for 24 h (Halami *et al.*, 1999). Zone of suppression of growth was assessed against the different antibiotic discs namely Amoxycillin(10µg), Cefaclor (30µg), Chloramphenicol (30 µg), Neomycin (30µg), Doxycycline (30µg), Vancomycin (30µg), Furazolidone (100µg), Clarithromycine (15µg), Oxacillin (1µg), Clindamycin (2µg), Penicillin–G (10µg), Erythromycin (15µg)

Diameter of inhibition zones were measured and results were expressed in terms of resistant(R), intermediate susceptibility (I), and suseptiblity (S) according to cut off levels proposed by Presscott *et al*.,(1999), NCCLS (2002).

Results and Discussion

There were different 77 isolates obtained using spread plate method from the curd and butter milk as a source. Further based on morphological, cultural and biochemical characteristics three isolates (SS-1, SS-2 and SS-3) were selected for probiotic study. These selected isolates were identified as bacilli and showed catalase test negative. None of the strains showed gas production. These indicate that all strains were homofermentative.

Further, effect of different salt concentrations and temperature on growth of isolates were studied. Results showed that selected three strains have good growth rate at the temperature 15°C, 37°C and 45°C (Table-1). All the selected three isolates were able to tolerate 1.0 -6.0 % NaCl concentration (Table 1), however SS-3 was able to tolerate NaCl concentration even up to 9%. Hoque *et al*., (2010) have also observed the NaCl (1-9%) tolerance of *Lactobacillus* sp. isolated from yoghurts.

Probiotic properties of Isolates

It was found that the isolates could survive nearly at pH 3.0 but all were inhibited at pH 2(Table-2, Figure 1). However amongst all isolates, SS-2 showed the highest resistance or tolerance at pH 3. Similar observations are also made by Sieladie *et al.*, (2011). According to results, all three isolates SS-1, SS-2 and SS-3 demonstrated good capacity to resist bile salts but SS-2 showed better resistance (Table 3, Figure 2). The gradual decrease in number of viable cells was observed when the concentration of bile salt was increased up to 1% (Klayraung *et al.*, 2008). *Lactobacillus* isolates showed resistance at 0.3 % (w/v) bile salt were considered as bile resistance strains (Vinderola and Reinheimer, 2003).

The antimicrobial effects of all the isolates against selected pathogenic bacteria *Escherichia coli, Staphylococcus aureus, Bacillus Subtilis, Proteus vulgaris were* studied because they are occasionally found as food borne microorganisms that might cause gastroenteritis. The results are shown in Table 4. All three isolates showed the higher antibacterial potential to all test organisms however SS-2 shows highest potency to all test organisms which indicates that the cell free solution of isolated *Lactobacillus* species were able to inhibit the growth of all the test microorganisms. Garriga *et al.* (1998) have reported inhibition of one or more enteric indicator strains (*E.coli, Salmonella e*nteritidis) by the *Lactobacillus paracasei* subsp. *paracasei.* However, *Lactobacillus acidophilus* strains isolated from infant faeces had weak antibacterial activity on *E.coli* and *Yersinia enterocolitica* (Xanthopoulous *et al.*, 2000). Daeschel (1989) has reported that the

antimicrobial effect of LAB is due to the production of lactic acid, reduction of pH, acetic acid, diacetyl, fatty acids, aldehydes and other compounds.

The antibiotic susceptibility of all three isolates was assessed by disc diffusion method using MHA medium and the results are shown in the Table 5. All the isolates were sensitive to antibiotic cefaclor, chloramphenicol, Doxycycline. The isolate SS-1 was resistant to Neomycin, Furazolidone, Erythromycin and the isolate SS-2 was resistance to Amoxycillin, Oxacillin, Clarithromycine while the isolate SS-3 was resistance to Neomycin, Furazolidone, Oxacillin and Penicillin – G. The antibiotic resistance traits among probiotic microorganisms are advantageous for survival in the gastrointestinal tract during antibiotic treatment.

Table.1 Characteristics of Lactic acid bacteria isolated Strains

Characteristic	SS-1	SS-2	SS-3
Gram stain reaction	+	+	+
Catalase reaction	-	-	-
CO_2 from glucose	-	-	-
Indole test	-	-	-
Methyl Red Test	-	-	-
Vogues Proskauer Test	-	-	-
Citrate Utilization test	-	-	-
Urease test	-	-	-
Growth at			
15°C		+	+
37°C		+	+
45°C		+	+
Growth in medium			
1% NaCl	+	+	+
2%NaCl	+	+	+
3%NaCl	+	+	+
4%NaCl	+	+	+
5%NaCl	+	+	+
6%NaCl	+	+	+
7%NaCl	+	-	+
8%NaCl	-	-	+
9%NaCl	-	-	+
Sugar Fermentation			
Lactose	+	+	+
Glucose	+	+	+
Mannitol	+	+	+
Fructose	+	+	+
Maltose	+	+	+

*Positive Reaction (+), Negative Reaction (-)

Table.2 Acid tolerance result-Absorbance at 620 nm by spectrophotometer

pH	SS-1	SS-2	SS-3
2	0	0	0
3	0.191	0.251	0.101
4	0.651	0.362	0.165
5	1.17	0.565	0.577
6	1.806	1.361	1.761
8	0.162	0.114	0.5

Table.3 Bile salt tolerance-Absorbance at 620 nm by spectrophotometer

Bile salt Concentrations	SS-1	SS-2	SS-3
0.05%	1.150	1.445	1.57
0.10%	1.111	1.345	0.98
0.15%	0.627	0.446	0.337
0.30%	0.043	0.394	0.307
0.50%	0.008	0.442	0.19
Without bile salt	2.067	2.368	1.809

Table.4 Antibacterial Activity

Isolates	Diameter of inhibition zone (mm)			
No	***E.coli***	***S. aureus***	***B. subtilis***	***P. vulgaris***
SS-1	20	17	19	15
SS-2	25	30	20	32
SS-3	20	19	18	14

Table.5 Lactic acid bacterial strains showing sensitivity/resistant with different antibiotics

Isolates	Antibiotics											
	AX	**CT**	**CH**	**NE**	**DX**	**ES**	**FX**	**SP**	**OC**	**ER**	**PG**	**CD**
SS-1	S	S	S	R	S	S	R	I	S	R	I	S
SS-2	R	S	S	S	S	I	S	R	R	S	S	I
SS-3	S	S	S	R	S	M	R	S	R	S	R	S

*AX- Amoxycillin(10µg), CT- Cefaclor(30µg), CH- Chloramphenicol(30 µg), NE- Neomycin(30µg), DX-Doxycycline(30µg), ES-Vancomycin(30µg), FX- Furazolidone(100µg), ST-Clarithromycine(15µg), OC-Oxacillin(1µg), CD-Clindamycin(2µg), PG- Penicillin – G (10µg) ER- Erythromycin(15µg); (R)- Resistant, (S)-Sensitive, (I) Intermediatory according to the performance standards for antimicrobial disc (Pathetes Biological Laboratories.) suspension tests.

Fig.1 Acid tolerance of Isolates

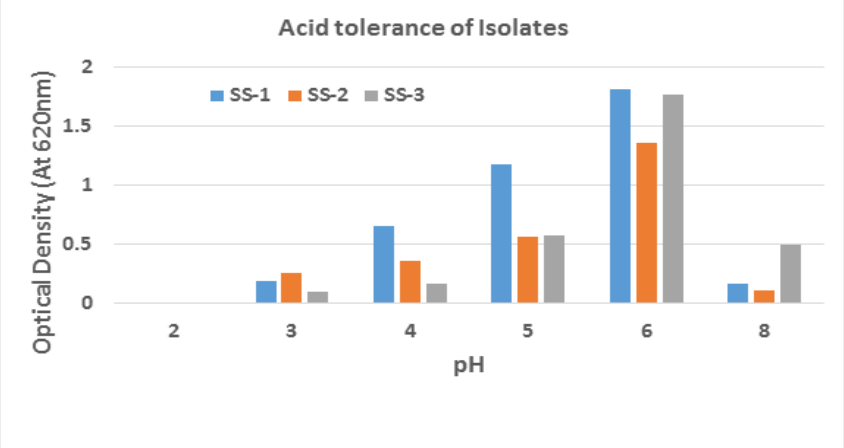
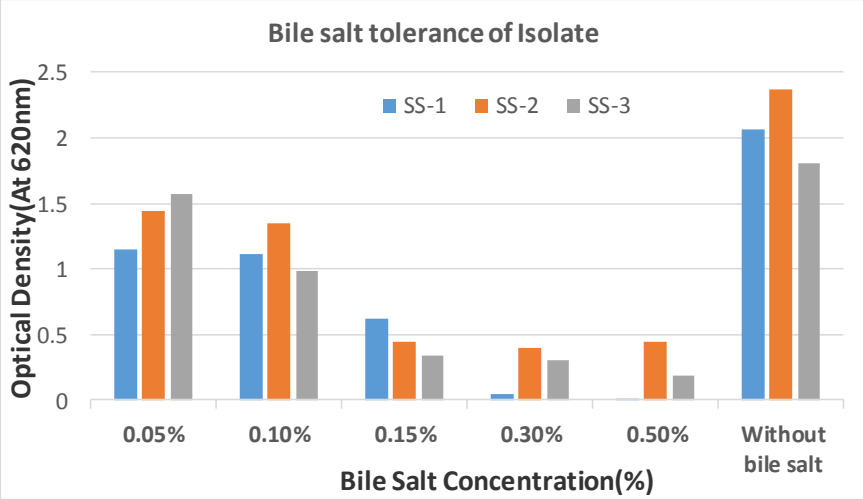


Fig.2 Bile Salt Tolerance



Such resistance to a wide spectrum of antibiotics indicated that if isolated probiotics induced in patients treated with antibiotic therapy may be helpful in faster recovery of the patients due to rapid establishment of desirable microbial flora. During co-administration of probiotics with antibiotics, probiotics should be resistant to certain antibiotics to survive in the gastrointestinal tract (Dixit *et al.*, 2013). Resistance of the probiotic strains to some antibiotics could be used for both preventive and therapeutic purposes in controlling intestinal infections (EI-Naggar, 2004). Results of antibiotic resistance/sensitivity of isolated strains are showing in Table – 5.

The present study results showed that the locally available dairy product Curd and buttermilk 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 characteristics study of the isolates may have potential for natural preservatives and may also be considered for probiotic

application or studies should be performed to use these isolates reliably for commercial applications.

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