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Assessment of Lactic Acid Bacteria Isolated from *Chakka*: An Indian Fermented Dairy Product

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Abstract

Objective: The objective of this study was to isolate, identify and characterize the lactic acid bacteria (LAB) from a domestic food (*Chakka*) and to evaluate their properties for application as starter cultures in fermented dairy products.

Methods: LAB were isolated using standard MRS/M17 media and characterized phenotypically and identified by 16SrDNA sequencing and Whole Genome Sequencing and their evolutionary relationships were plotted using MEGA X. Culture conditions were optimized in a shaker incubator and different media formulations using Skimmed Milk Powder, Yeast Extract, Glucose, and Casein Enzyme Hydrolysate were designed and tested. Subsequently, the isolates were grown in a 1 L bioreactor under controlled conditions to study their total viable count and the growth rates. Lactic acid production was quantified in MRS and laboratory formulated M2 media under optimized growth conditions. The recovery, quantification and total yield of exopolysaccharides (EPS) produced by the isolated strains was also done.

Results: The isolates were grouped as bacilli (C2, C4, C5, C12, and P1) and cocci(Q1, and Q2). The bacilli were identified as subspecies of *Lactobacillus delbrueckii* and cocci were identified as *Streptococcus thermophilus*. M2 media was found to be a viable alternative to standard media. Bacilli and cocci were found to produce L and D-lactic acid, respectively, and bacilli were better lactic acid producers than cocci. Similarly, bacilli were found to be better EPS producers in M2 media.

Conclusion: The results suggest of this study suggest that strains isolated from *Chakka* could be potential starter cultures for use in yogurt manufacturing. The critical understanding about the metabolism of these LAB would be helpful for the maintenance of strains and their exploitation as starter cultures.

Keywords: Lactic acid bacteria; Starter cultures; Fermented foods; *Lactobacillus delbrueckii subsp. indicus*; 16s-rRNA sequencing; MEGA X; Media optimization; Exopolysaccharides

Introduction

'Functional' dairy products are ideal vehicles for delivering a defined number of live, beneficial bacteria to the human gut. Since times immemorial, they have been central in the manufacture of various fermented foods like yogurt, cheeses, fermented milk, vegetables, meat, wine and therefore, are heavily used as starters in the food industry [1-5]. Therefore, lactic acid bacteria (LAB), producers of lactic acid as their primary metabolic product, are diversely applied organisms in the food industry. Their extended and safe use as starter cultures in fermented products qualifies them as generally recognized as safe (GRAS) microorganisms [6].

LAB is a heterogeneous group of spherical (cocci) or rod-shaped (bacilli), non-sporulating, non-motile gram-positive, catalase negative, anaerobic, and aero-tolerant bacteria [7]. They are non-pathogenic [8], and can be homo-fermentative, facultative homo-fermentative or hetero-fermentative. They can metabolize mono- and disaccharides like glucose and lactose to harness energy. Using beta-galactosidase enzyme, homo-fermentative LABs hydrolyze lactose in the milk and produce two moles of lactate and ATP by Entner-Meyerhof-Parnas (EMP) pathway [9].

Besides lactic acid, these bacteria produce a variety of compounds like acetic acid, ethanol, aroma compounds, enzymes, exopolysaccharides (EPS) [2]. Low pH, the non-dissociated acid, and production of antimicrobial metabolites called bacteriocins also contribute to their antagonistic property [9,10]. They are acid tolerant and bile tolerant[8], making them suitable for the food industry; although presence of the 'inhibitory' secondary metabolites[11], and additional stresses based on the technique used for their preservation, such as spray drying [12], freeze drying [13], and fluidized bed drying [14] may restrict their applicability.

Recently, LAB derived EPS have gained popularity due to various reasons. Their ability to improve rheology (viscosity and elasticity) makes them excellent candidates as natural gelling agents, bio-thickeners, emulsifiers, and physical stabilizers to bind water and limit syneresis [15]. Although EPS are themselves tasteless, they prolong the time that the milk product spends in the mouth, enhancing its taste through improved volatilization of the intrinsic flavor in the product [16] and contribute to the organoleptic quality of the fermented foods, in texture and taste perception, mouth-feel, and stability [15,17]. Also, due to increased viscosity, the foods may remain in the gastrointestinal tract for a longer duration and therefore be beneficial to the transient colonization by probiotic bacteria [18]. Various factors such as bacterial growth, phase, medium composition (carbon and nitrogen source), pH, and temperature influence their production [15]. Also,

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LAB synthesized EPS appear to have anti-tumor, anti-ulcer, immuno-modulating, and cholesterol-lowering activity [14].

In Maharashtra and Gujarat (India), the fermentation of milk into curd and straining and separation of the whey to get the concentrated curd, called '*Chakka*' is a routine in households and dairies. *Chakka* is used as a base for making Shrikhand, a popular culinary concept. The preparation of Shrikhand involves mixing *Chakka* with the required amounts of sugar, color and flavor to get the product. This thick curd marinade has an exciting, sour-sweet taste and holds great economic potential. Therefore, this study aimed to isolate, identify, and characterize the naturally occurring LABs from *Chakka*, to assess their potential as starters. The study also included the optimization of culture media and other parameters and evaluation of lactic acid and exopolysaccharides produced.

Materials and Methods

Chemicals

All chemicals were purchased from Qualigens, India and HiMedia, India and were of analytical grade and were used directly without further purification. M17 Agar and De Man Rogosa Sharpe (MRS) agar were obtained from HiMedia, India and Accumix, India, respectively.

Isolation and purification of cultures

All the strains were isolated from the *Chakka* prepared using pasteurized full cream milk, obtained from M/s. B.G. Chitale Dairy at Bhilawadi in Sangli District, Maharashtra, India. The LAB isolates were purified according to the method adopted [19]. The plates were incubated in micro-aerobic conditions (5% CO_2) [20], at 37°C for mesophilic and at 42°C for the thermophilic LAB for 48 hours. All experiments were performed in triplicate. The colonies were randomly selected from the agar surface and were purified by streaking twice on fresh MRS [21] and M17 [22] agar. Colonies were selected according to their morphology. The purity of isolates was checked by four streak method on the respective media followed by macroscopic and microscopic examination. The strains displaying the general characteristics of lactic acid bacteria were chosen from each plate for further studies.

The strains were stored without appreciable loss of properties in reconstituted sterile skimmed milk at -20°C. Working cultures were also kept on MRS or M17 agar slants at 4°C.

Phenotypic characterization of isolates

The cultures were characterized based on differentiation scheme for genera level identification of lactic acid bacteria and profile matching method based on Bergey's manual of systematic bacteriology [7]. The individual colonies were subjected to preliminary screening by examination for cellular morphology, the colony appearance, were Gram stained, observed for motility, and tested for cytochrome oxidase and catalase [23]. Hugh and Leifson's test, which allows determination of ability of an organism to act as oxidizer or fermenter under aerobic and anaerobic conditions was performed in Oxidizer/Fermenter (O/F) medium [24]. The isolates were also tested for acid production from carbohydrates 1% (w/v) with glucose, sucrose, lactose, arabinose, maltose and sorbitol.

The strains were analyzed for their ability to grow at different temperatures (15°C, 25°C, 40°C and 45°C) and in various pH values (pH 4, 5, 6, and 7) in MRS and M17 broth. Growth of isolates in the presence of different NaCl concentrations was done by incubating the isolates in 2, 3, 4 and 6% of NaCl in MRS broth and M17 broth at pH 6.5 and 7.0, respectively. The isolates were grown under three different conditions to probe the requirement of oxygen for growth of different isolates: in the presence of low Oxygen (5% CO_2 in an anaerobic incubator), absence of oxygen (anaerobic jar) and aerobic conditions at the same temperature. Spread plate technique was used for the viable count [25,26].

Glycerol stocks of the screened isolates were prepared by mixing 1mL of 40% glycerol (HiMedia, India) with 1 mL of the cell pellet in M17 or MRS broth and stored at -80°C in 2 mL Cryoprotectant vials (HiMedia, India). A set of MRS and M17 slants were also made and stored at 4°C for use as working culture.

Molecular Characterization by Sequencing and Phylogenetic analysis

16s-rRNA sequencing: Selected isolates with desired biochemical and other characteristics were submitted for identification by 16s rRNA gene sequencing at geneOmbio technologies Pvt. Ltd. Pune. India. The 16s-rRNA gene was amplified in a standard PCR reaction with the primer pair 27F(5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R(5'-TACCTTGTTACGACTT-3') and sequenced using 3130 Genetic analyzer and assembled using ChromasPro v3.1 [27]. The sequences were analyzed using the Sequencing Analysis 5.2 software. BLASTn analysis was performed with Megablast program.

Whole genome sequencing: The identified isolates were sent for whole genome sequencing at Nirav Bio Solutions Pvt. Ltd. The genome sequences were assembled and CDS was predicted using gene mark software. rRNA and tRNA were predicted using RNAMMER and tRNAscan tools, respectively. The genome annotation was performed using NCBI Blast & Blast2GO. Results for all analyses were provided in standard format.

Evolutionary analysis by Maximum Likelihood method: The evolutionary history was inferred by using the Maximum Likelihood method and the Kimura 2-parameter model [28]. The model with the lowest Bayesian information criterion (BIC) is said to describe the substitution pattern the best, and therefore, such a model was selected using MEGA X [29], which was used for all the evolutionary analyses. For each model, MEGA X also provided AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (lnL), and the number of parameters (including branch lengths) [30], estimates of gamma shape parameter and/or the estimated fraction of invariant sites (+I), assumed or estimated values of transition/transversion bias ® followed by nucleotide frequencies (f) and rates of base substitutions (r) for each nucleotide pair. For determining ML values, a tree topology was automatically computed [29].

The bootstrap consensus tree was inferred from 1000 replicates and is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [31]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log-likelihood value. Non-uniformity of evolutionary rates among sites was modeled by using a discrete Gamma distribution to obtain evolutionary rate differences among sites (5 categories (+G, parameter=0.0500 for cocci and 0.1594

for bacilli)). The evolutionary analysis involved 20 nucleotide sequences for bacilli and nine sequences for cocci. The sequences were obtained from 16s-rRNA sequencing data for the laboratory isolates, and the rest were downloaded from NCBI Supplementary Table 1.

Growth curve of isolates in a bioreactor

Growth curves were plotted to find out the different growth phases in the Bioreactor vessel (Eppendorf BioFlo 120, 1 L capacity) in batch mode. The vessel containing 800 mL of MRS broth at pH 6.7 or M17 broth at pH 7.0 was sterilized at 121°C for 15 mins. The pH electrode (METTLER TOLEDO, L120 mm) was calibrated before sterilization. The bioreactor was cooled to room temperature and agitator, temperature sensor, DO sensor, heating coil, and sparger line through 0.2 µm filter connections to the console were made. DO probe calibration in bioreactor vessel was done. Bioreactor conditions for growth curve are shown in Table 1. The optical density of the cell mass was measured at 600 nm using spectrophotometer (LABMAN) and viable counts performed in MRS or M17 agar in duplicates at 37°C for 48 hours. Viability of isolates was checked by spread plate method on MRS and M17 agar as described [25,26].

Media Optimization in the bioreactor

In order to improve the biomass yield at low cost, and preserving original biochemical characteristics of isolates, various milk-based media were designed and tested Table 2. Based on the results of viability, media residue left after centrifugation (at 2415 g for 10 min), quantity of cell pellet by centrifugation, and economic constraints, a medium was chosen for subsequent experiments. Controlled bioreactor conditions for all four media were shown in Table 1. Samples were drawn from continuously agitated broth at regular intervals. Agitation was slightly increased at the time of sampling to allow homogeneity in the culture broth.

The samples were analyzed by using a Spectrophotometer by measuring optical density at 600 nm for M17 or MRS broths and 640 nm for milk-based media. For milk-based broth, 1 mL of broth sample was dissolved in 9 mL of 0.6% EDTA (HiMedia, India) solution (pH 12.0) [32] and OD reading was measured at 640 nm on the spectrophotometer. In all cases, the cells were harvested at early stationary phase, indicated by reduction in base feed pump output and a plateaued absorbance reading.

Estimation of lactic acid production and isomerism

The production of lactic acid was determined by titrating 10 mL homogenized samples against 0.25 M NaOH, using 1 mL phenolphthalein indicator (0.5% (w/v) in 50% ethanol). The total titratable acidity was calculated as a percentage of lactic acid (v/v). Each mL of 1 N NaOH is equivalent to 9.008 mg of Lactic acid [33]. Lactic acid isomerism was checked by D/L Lactate assay kit of Mega enzyme [34,35].

Exopolysaccharide estimation

EPS estimation was done as described [36] with slight modifications using the phenol-sulfuric acid method [37]. In order to determine the polymer dry mass, remaining sample was transferred to an empty petri plate and was dried in a Hot air oven with temperature 105°C. After every 2 h, it was weighed, until a constant reading was observed [38].

Observations and Results

Isolation and phenotypic characterization of the isolates

A total of 7 bacterial cultures were isolated from *Chakka* sample and labeled as Q1, Q2, C2, C4, C5, C12, and P1. Biochemical characterization of all the isolates was done, and their characteristics were noted Table 3 and Supplementary Table 2. All the isolates were found to be Gram-positive, catalase and cytochrome oxidase negative, non-motile cocci or bacilli in long chains. C2, C4, C5, C12, and P1 were rod-shaped whereas Q1 and Q2 were cocci shaped.

Hugh and Leifson's test showed an anaerobic process of fermentation; specifically, facultative anaerobic fermentation. All isolates utilized arabinose, lactose, glucose, maltose, sucrose, and

Parameters	For Growth curve		For media optimization	
	For Streptococcus spp.	For Lactobacillus spp.	For Streptococcus spp.	For Lactobacillus spp.
Temperature [in °C]	40	40	40	40
Aeration [in Standard Liter Per Minute (SLPM)]	0.1-0.2	0.1-0.2	0.1-0.2	0.1-0.2 SLPM
Gas Mixture (Air:Nitrogen:CO_2)	90:10:0	0:90:10	90:10:0	0:90:10
Agitation [in Rotations per Minute (RPM)]	50-100	25-30	50-100	25-30
Inoculum Size [(18 hours old having viable count ~10^8 CFU/mL) in%]	2	2	5	5
Dissolved Oxygen level [in%]	Below 50	Below 10	Below 50	Below 10
Optimum pH for growth	5.7-5.8	5.7-5.8	5.7-5.8	5.7-5.8
Base used for maintenance of pH	20% NH_4OH		20% NH_4OH	

Table 1: Bioreactor operating parameters: The bioreactor was operated under different conditions for bacilli and cocci separately for the two experiments *viz*. Growth curve and media optimization.

Composition	Media Composition			
	M1	Industrial Whey	M2	MRS And M17
Skimmed milk powder	2%	Residual industrial whey	2% (hydrolyzed)	MRS (Accumix) and M17(HiMedia)
Yeast extract	0.1%	0.1%	1%	
Glucose	0.1%	0.1%	2%	
Casein enzyme hydrolysate	0.3%	0.3%	-	
pH, after autoclave	6.5	6.3	6.7	6.5
Colour of media	clear, caramel	Turbid, brown	Clear, light brown	Clear, brown

Table 2: Composition of various media: Media designed using different raw materials such as Skimmed Milk Powder, Yeast Extract, Glucose, Casein Enzyme Hydrolysate, and Residual Industrial Whey were tested for their efficacy against the standard M17 and MRS media. This table lists the composition of the different media used.

sorbitol as a carbohydrate source and produced D/L-lactic acid. All the isolates showed growth in 25-45°C whereas no growth at 15°C. All strains except C4 showed growth in 2% NaCl whereas only Q2 showed growth up to 4% NaCl. Bacilli showed growth in a wider pH range 4-7 as compared to a narrower range of 6-7 displayed by cocci.

Molecular identification

All the isolates were identified to sub-species level by 16s-rRNA sequencing and classified in two genera: Streptococcus and Lactobacillus Table 4. The genome characteristics of the identified strains are summarized in Supplementary Table 3. Phylogenetic analysis revealed that the laboratory strains Q1, Q2, C2, P1, and C4 clustered around the respective subspecies sequences obtained from NCBI. C5 and C12 were found to be clustered together, separately from the other subspecies. In case of call the strains, divergence was observed as the differences in the nucleotide sequences increased (Figure 1).

Growth curve

A typical bacterial growth curve was observed for all the isolates grown in the bioreactor. Viable counts of LAB in various media varied from 10^8-10^{10} CFU/mL. The growth curves for all isolates showed optimum mid log phase at pH 5.5-5.7, with viable count in the range of 10^8-10^{10} in 120-240 min (Figures 2 and 3).

Media optimization in the bioreactor

In this experiment, pH, Glucose and Yeast Extract were found to be crucial factors for optimization of media. The observations are summarized in Table 5. The viable counts observed for both the standard media (MRS/M17) and designed media were comparable, whereas the volume of the concentrated cell pellet varied considerably. The media containing industrial whey gave the most unsatisfactory results, possibly due to already accumulated inhibitory secondary metabolites in the pH reconstituted whey. Although M1 fared better in terms of cell pellet volume as compared to M2 w.r.t. the standard media, it contained an expensive component CEH,m which would have been a recurring cost for large scale fermentation.

M2 did not have CEH and proved to be more economical. Also, both the milk-based media had a significant disadvantage-accumulation of milk solids as a pellet after the autoclave step in case the pH got disturbed or at the end of the incubation period. The pellets gave erroneous concentrated cell pellet volume and interfered with the viable count. Also, the milk solids made it challenging to obtain a cell

Isolate/Characteristics		C2	C4	C5	C12	P1	Q1	Q2
Physiological characterization (+: growth, -: no growth)								
Survival at different pH	4.0	-	-	-	-	-	-	-
	5.0	+	+	+	+	+	-	-
	6.0	+	+	+	+	+	+	+
	7.0	+	+	+	+	+	+	+
	8.0	-	-	-	-	-	-	-
Survival at different temperatures [in °C]	15	-	-	-	-	-	-	-
	25	+	+	+	+	+	+	+
	37	+	+	+	+	+	+	+
	40	+	+	+	+	+	+	+
	45	+	+	+	+	+	+	+
Survival in different NaCl%	2%	+	-	+	+	+	+	+
	4%	-	-	-	-	-	-	+
	6%	-	-	-	-	-	-	-
Biochemical tests (+: presence of the enzyme, -: absence of the enzyme)								
Catalase		-	-	-	-	-	-	-
Oxidase		-	-	-	-	-	-	-
Carbohydrate utilization (Yellow: presence of acid production)								
Arabinose		Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Glucose		Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Sucrose		Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Maltose		Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Sorbitol		Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow

Table 3: Phenotypic characterization of the bacterial isolates: Physiological characterization of all strains based on their survival at different pH, temperatures, and concentrations of NaCl along with their biochemical characterization by testing for oxidase, and catalase and their growth in different sugar substrates (Arabinose, Glucose, Sucrose, Maltose, and Sorbitol) is summarized in this table. Note: Supplementary Table 3 provides information about the Gram reaction, motility test, and growth under aerobic and anaerobic conditions.

Sr. No.	Laboratory Code	Name
1	C2	*Lactobacillus delbrueckii sub sp. indicus*
2	C5	*Lactobacillus delbrueckii sub sp. indicus*
3	P1	*Lactobacillus delbrueckii sub sp. indicus*
4	C4	*Lactobacillus delbrueckii sub sp. bulgaricus*
5	C12	*Lactobacillus delbrueckii sub sp. lactis*
6	Q1	*Streptococcus thermophilus*
7	Q2	*Streptococcus thermophilus*

Table 4: Names of identified isolates:

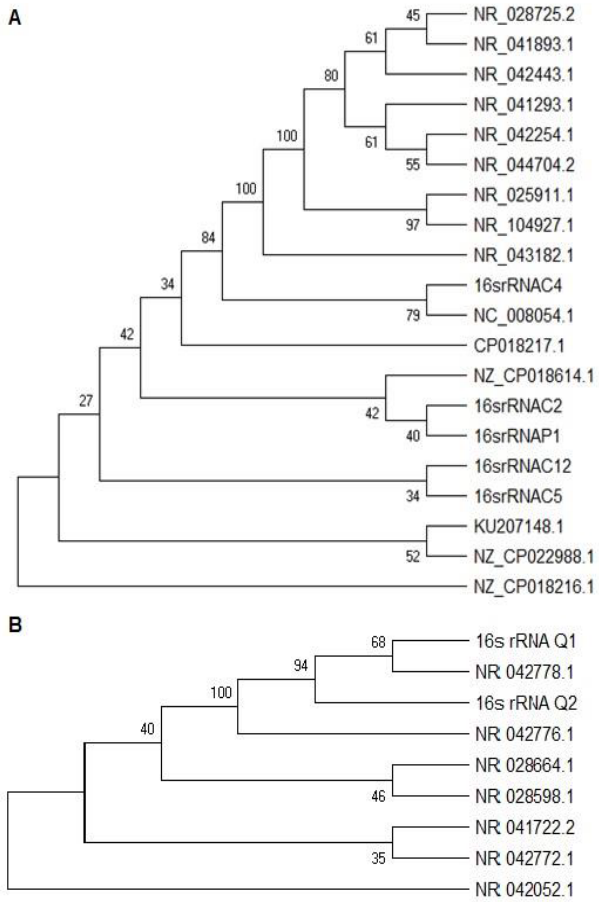

Figure 1: Evolutionary relationships between taxa. A) The relationships between different isolated bacilli and those obtained from NCBI. B) The relationships between isolated cocci and those obtained from NCBI.

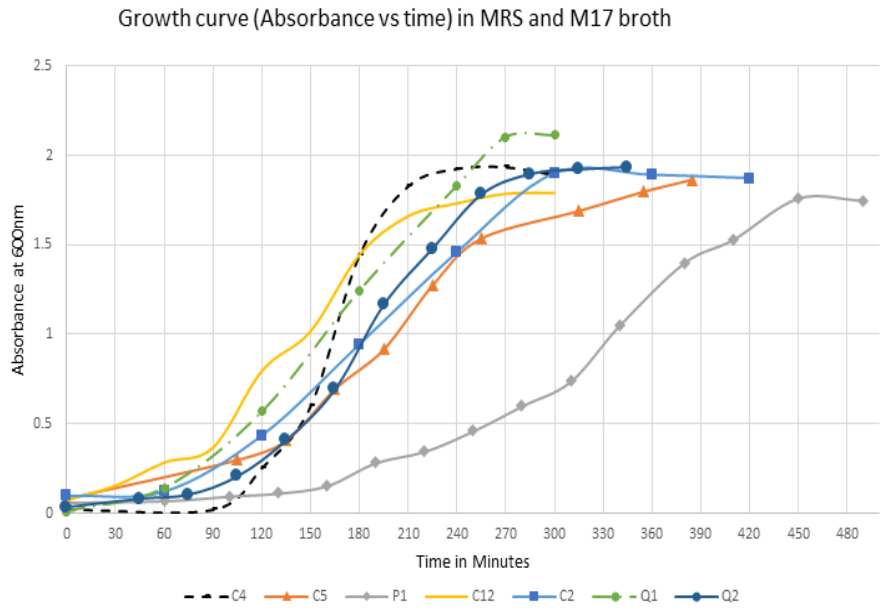

Figure 2: Growth curve (Absorbance vs. time) in MRS and M17 broth: For both the broths, the absorbance was measured at 600 nm. A typical bacterial growth curve was obtained for all the strains. P1 showed slowest growth as compared to other cocci and bacilli.

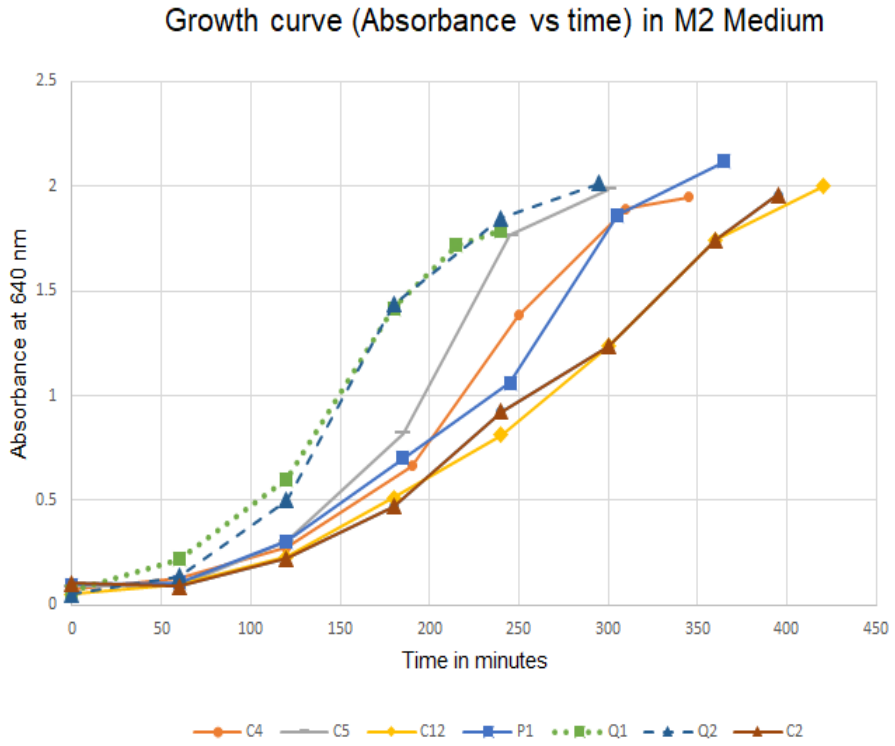

Figure 3: Growth curve (Absorbance vs time) absorbance in M2 broth: The absorbance was measured at 640 nm. A typical bacterial growth curve was obtained for all the strains. C12 showed slowest growth as compared to other cocci and bacilli.

Name of Media	M1	Industrial whey	M2	MRS/ M17 broth
Observation after Centrifugation step	Brown pellet (insoluble) and white cell pellet at bottom with milky white supernatant	White cell pellet at bottom and clear brown supernatant	White cell pellet at bottom and clear brown supernatant	White cell pellet at bottom and Clear brown supernatant
Viability count [in log CFU/mL]	~10^{10}	~10^{9}	~10^{10}	~10^{10}
Concentrated cell pellet volume [in mL]	~30	< 5	~25	~25

Table 5: Media optimization observations and results: The table reports the observations for the media optimization experiment before and after growing the LAB in the media.

pellet at the centrifugation speeds suitable for cells. These problems were successfully solved by hydrolyzing M2 media by using food-grade citric acid powder before autoclave and using the filtered hydrolysate to make the media.

Estimation of lactic acid production and isomerism

All the strains produced Lactic acid in M2 and MRS/M17 broth after 24-hour incubation (Figure 4). The strains C4 and C5 showed the highest level of lactic acid production whereas Q1 and Q2 showed comparatively lesser lactic acid production. Also, the strains Q1 and Q2 produced D-Lactic acid, whereas C2, C4, C5, C12 and P1 produced L-Lactic acid in M2 and MRS/M17 media.

Exopolysaccharide estimation

After 24-hour incubation, exopolysaccharide production in M2 broth was analyzed and all the isolates produced exopolysaccharides in the range of 300-600 mg/L (Figure 5).

Conclusion

Dairy industry is always on the lookout for novel starter cultures with better properties. In our study, we have attempted to provide a basis for such a starter culture using unexplored strains such as *Lactobacillus delbrueckii subsp. indicus*. The organisms we isolated were from traditional sources and were grown in an economic media with comparable viability and titratable acidity to standard media. Media and growth conditions optimization in a bioreactor revealed a standard set of conditions for each isolate, which were in concordance with those required for dairy starter cultures. The isolates were phenotypically characterized and identified using genome sequencing and most of them were found to have concordant evolutionary relationships with the strains obtained from NCBI. Overall, this study provides important information to make a commercial starter culture using *Lactobacillus delbrueckii subsp. indicus* apart from other strains. In future, our objective is to optimize the production at larger bioreactor volumes and formulation of the starter culture to ensure high viability and titratable acidity upon long term storage.

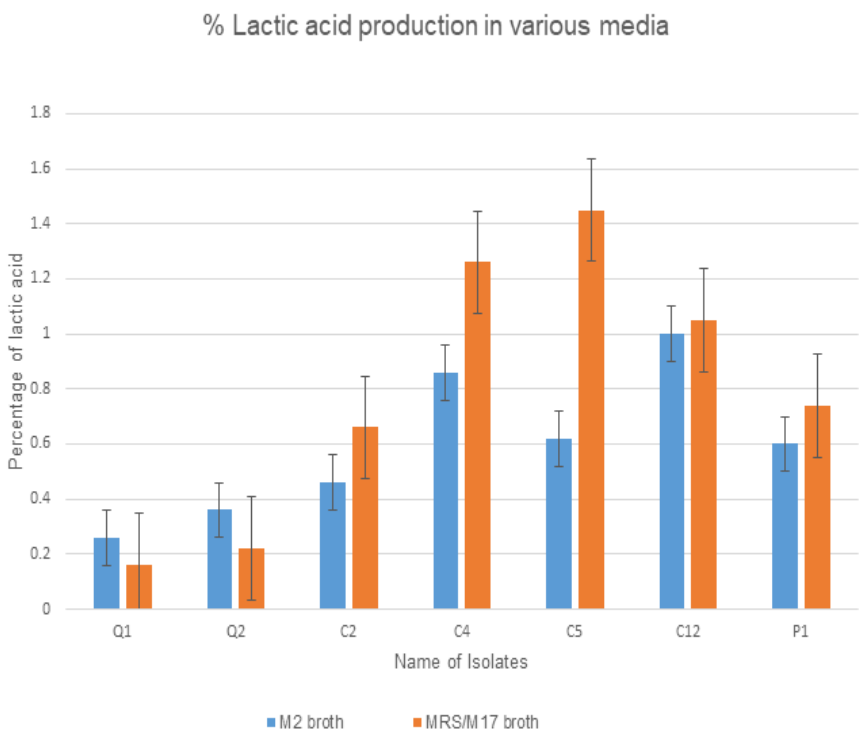

Figure 4: Percentage of Lactic acid produced in M2 and MRS/M17 broth: Bacilli (C2, C4, C5, C12 and P1 were found to be better producers of lactic acid as compared to cocci (Q1 and Q2). Slightly better production of lactic acid was observed in standard media as compared to M2.

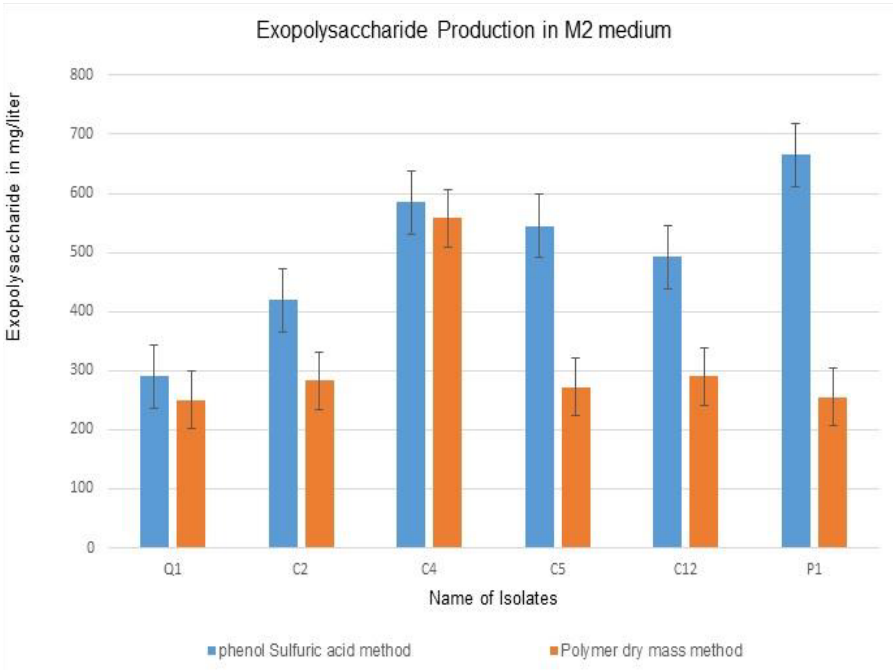

Figure 5: Exopolysaccharide produced in M2 medium: Bacilli (C2, C4, C5, C12 and P1 were found to be better producers of EPS as compared to cocci (Q1).

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