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RESEARCH ARTICLE

Production of low browning Mozzarella cheese: Screening and characterization of wild galactose fermenting *Streptococcus thermophilus* strains

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Abstract

Streptococcus thermophilus is a thermophilic lactic acid bacterium used for the manufacture of yoghurt and cheese. Most strains of *S. thermophilus* were galactose negative (Gal^-) due to the weak induction of *gal* operon. Galactose positive (Gal^+) strains can be exploited in reducing problems like browning of Mozzarella cheese. In this study, six galactose positive lactic *Streptococcus* spp. submitted to National Collection of Dairy Cultures (NCDC) were screened for galactose fermentation and characterized. Out of six isolates, four isolates namely NCDC 659, NCDC 660, NCDC 527, NCDC 661, were confirmed as *S. thermophilus* by phenetic and molecular methods. Galactose fermentation of these isolates in J8 broth containing 1% galactose revealed that NCDC 659 utilized 0.44% of galactose, followed by NCDC 661 (0.36%), and NCDC 660 (0.29%). In milk fermentation, NCDC 659 released less galactose (0.24%), followed by NCDC 661 (0.34%) and NCDC 660 (0.39%) after 8 h. Gal^+ isolates exhibited enhanced stability in galactose broth than in milk after ten sub-cultures. During Mozzarella cheese preparation with Gal^+ *S. thermophilus* strains and Gal^+ *Lactobacillus helveticus* (NCDC 292), Gal^+ *S. thermophilus* NCDC 659 expelled less galactose (0.15%) followed by NCDC 661 (0.3%) and NCDC 660 (0.33%), compared to the reference strain. Also, browning was less in Gal^+ NCDC 659 and showed a hunterlab (L*) value (76.09±0.37) than control Gal^- NCDC 218 (66.68±0.4). This study revealed that Gal^+ *S. thermophilus* strains possessed more stability and it can be paired with Gal^+ *L. heveticus* NCDC 292 for developing low browning Mozzarella.

Introduction

Streptococcus thermophilus, is an important bacterium used in the food industry for the manufacture of yoghurt and Swiss, Brick, Parmesan, Provolone, Mozzarella and Asiago cheeses (Hols et al., 2005). It ranks 2nd, among the industrial dairy starters after *Lactococcus lactis.* The thermophilic bacteria *S. thermophilus* contain a highly homologous *lacSZ* operon. It contains the β-galactosidase (*lacZ*) gene located downstream from the *lacS* gene encoding a lactose permease (*lacS*), which belongs to the galactoside–pentose hexuronide translocators. This β-galactosidase (*lacZ*) gene of *S. thermophilus* is used for its species-specific identification by polymerase chain reaction (PCR) based methods (Lick et al., 1996).

S. thermophilus is highly adapted to lactose which is transported by permease *lacS* (Foucaud and Poolman, 1992). Although lactose is efficiently transported and hydrolyzed intracellularly, most strains of *S. thermophilus* do not utilize galactose and

only ferment the glucose moiety of lactose, while the galactose portion of lactose is excreted back into the medium in a stoichiometric proportional amount of lactose uptake. However, the genes necessary for galactose catabolism are present in all *S. thermophilus*, but they are not expressed in all the strains. The genes for galactose utilization and its control have been reported to be organized in the *galR-galKTEM* gene cluster located upstream of the *lac* operon in *S. thermophilus* strain CNRZ302 (Vaillancourt et al., 2002, 2008). The inability of *S. thermophilus* to utilize galactose may be due to either the absence of one or more catabolic enzymes or the absence of a galactose transport system (Hutkins et al., 1985). Accumulation of galactose in dairy products leads to several undesirable effects, especially browning of Mozzarella cheese (Kindstedt and Fox, 1993), production of CO_2 by nonstarter bacteria (Thomas et al., 1980) and growth of spoilage microorganisms (Tinson et al., 1982). The presence of an excess amount of galactose in dairy products may also adversely affect human health, particularly in individuals with galactosemia (Novelli and Reichardt, 2000).

Mukherjee and Hutkins (1994) isolated Gal^+ *S. thermophilus* from cheese and yogurt samples by plating on Elliker-galactose medium containing 0.5% (w/v) galactose and 40 mg L^{-1} Bromo cresol purple. Gal^+ variants were also derived from wild type Gal^- *S. thermophilus* CNRZ 302 in Gal-M17 media (Vaughan et al., 2001; van den Bogaard et al., 2004). De Vin et al., (2005) investigated 49 *S. thermophilus* from different sources like yogurt and cheese and found 8 galactose-positive phenotypes in M17 broth containing 0.5% (w/v) galactose as the sole carbon source.

Low moisture part skim (LMPS) Mozzarella is consumed worldwide in pasta filata-type dishes. *S. thermophilus, Lactobacillus bulgaricus* and *Lactobacillus helveticus* are commonly used in the manufacture of Mozzarella. Mozzarella is used primarily in the preparation of pizza and hence it is called pizza cheese. Stretching, oiling off, melting, and browning are considered to be the most important characteristics of Mozzarella cheese, by pizza manufacturers (Kindstedt and Fox, 1993). Browning (Maillard reaction) of cooked Mozzarella cheese during pizza making (>260°C) is mainly a result of residual lactose or galactose in cheese that reacts with peptides and amino acids. Galactose was found to be more reactive than lactose and glucose (Johnson and Olson, 1985). This can be problematic because excess browning makes pizza disagreeable to the consumer. The Maillard reaction in cheese is mainly dependent on the proteolytic activity as well as lactose and galactose utilizing abilities of starter cultures used in cheese. Since most of the strains utilize the glucose portion of lactose and expels galactose, which promotes browning.

Browning can be controlled by several strategies viz., by manipulating cheese-making condition through washing of curds, which will minimize the levels of lactose and galactose and selecting suitable non-browning starter cultures (Gal^+). Mozzarella cheese made with galactose-fermenting (Gal^+) strains of *S. thermophilus* has reduced capacity for browning (Hutkins et al., 1986; Bley et al., 1985; Johnson and Olson, 1985). Oberg et al., (1991) further suggested that a non-proteolytic Gal^+ strain of *L. helveticus* would be best for reducing the brown color. During aging, non-proteolytic strains would produce fewer amino groups than proteolytic strains and presumably cause less browning. Hutkins et al., (1985) suggested that galactose-fermenting *S. thermophilus* may have limited potential when used as single strain starter in Swiss cheese but may be useful when combined with galactose positive *Lactobacillus* in the manufacture of Mozzarella cheese. Mozzarella cheese made with galactose-positive (Gal^+) cultures of *S. thermophilus* and *L. helveticus* had lesser browning during cooking. They also found that browning increased with levels of residual galactose (Johnson and Olson, 1985).

As the complete utilization of galactose is a desirable property in various dairy products, there is a requirement of galactose fermenting *S. thermophilus* in order to overcome the problems associated with galactose negative strains. The objectives of this study were to screen and characterize galactose fermenting strains of *S. thermophilus* and to analyze the galactose utilization in broth and milk by the Gal^+ strains. The screened Gal^+ strains were then used as starter cultures in Mozzarella cheese making and the levels of galactose remaining in cheese were evaluated by comparing with commercial Gal^- cultures.

Materials and Methods

Bacterial strains and culture conditions

Six thermophilic galactose positive lactic *Streptococcus* strains (AJM, AUKD8, JM1, KM3, HRL and SRC) isolated from fermented milk and plants submitted to the National Collection of Dairy Cultures (NCDC), National Dairy Research Institute, Karnal, Haryana under accession no, NCDC 527 (AUKD8), NCDC 547 (HRL), NCDC 572 (SRC), NCDC 659 (AJM), NCDC 660 (JM1) and NCDC 661 (KM3) were examined. In this study Gal^- *S. thermophilus* NCDC 218 was used as the reference strain. Strains were maintained as frozen stocks at -20°C in the presence of 15% glycerol as a

cryoprotective agent. Frozen cultures of Streptococcal spp. were routinely transferred to J8 broth supplemented with 0.5% (w/v) lactose for Gal^- strains and with 0.5% (w/v) galactose for the Gal^+ strains and grown at 42°C. Before each experiment, the cell density of all cultures was adjusted to 0.01 absorbance at 660 nm.

Confirmation of *S. thermophilus* for galactose fermentation

Galactose fermentation in Streptococcal isolates were confirmed by streaking in Elliker agar containing 0.5% galactose and 40mg/l Bromocresol purple and incubated at 42°C for about 24 to 48 h (Mukherjee and Hutkins, 1994).

Preliminary identification of the strains

Strains were presumptively identified as *S. thermophilus* by morphological, physiological and biochemical tests such as gram staining, catalase test, growth at different temperatures (15°C and 45°C), production of CO_2 from glucose, growth at different concentration of NaCl (2.5 to 4.0 %), bile esculin hydrolysis and heat treatment at 60°C for 30 min (Hardie, 1986). In addition, sugar fermentation strips viz., API50CHL and API20Strep (BioMérieux, Lyon, France) were used for further confirmation of *S. thermophilus* strains.

Genotypic characterization of Streptococcal isolates DNA extraction from pure cultures

Streptococcal genomic DNA was isolated as described by Pospiech and Neumann (1995). The quantity and quality of the isolated DNA was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and it was stored at -20°C.

Species specific PCR

The identification of isolates was performed by the species-specific PCR using primers targeting lacZ gene of *S. thermophilus* with sequence F: 5′-CACTATGCTCAGAATACA-3′and R:5′-CGAACAGCATTGATGTTA-3′as described by (Lick et al., 1996). *S. thermophilus* NCDC 218 was used as positive control.

Cloning and sequencing of 16S rDNA gene from *Streptococcus* PCR amplification of 16S rDNA gene

PCR amplification of the 16S rDNA gene from each Gal^+ isolates was performed using MJ Mini™ Personal Thermal cycler, Bio-Rad (Hercules, California, USA). The universal oligonucleotides primers P27F 5'-AGA GTT TGA TCC TGG CTC AG-3' and and P1492R 5'-GGC TAC CTT GTT ACG ACT T-3'were used for the amplification of bacterial 16S rRNA gene (Chun and Goodfellow, 1995). The PCR program consisted of an initial denaturation for 4 min at 94°C, followed by amplification of 30 cycles comprising of denaturation for 45 sec at 94°C, annealing for 30 sec at 56°C and extension for 1.0 min at 72°C. PCR was completed with a final elongation step of 7 min at 72°C. The resulting amplicons were separated on 1.5% agarose gel, followed by ethidium bromide staining and the bands were visualized under AlphaImagerEP (AlphaInnotech Corp, Santa Clara, CA, USA). The size of DNA fragments were estimated using a GeneRuler™ 1Kb DNA ladder (Fermentas UAB, Vilnius, Lithunia).

Purification and cloning of amplified 16S rDNA

The bacterial 16S rDNA amplicons derived from PCR with P27F and 1492R primer pair was purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In order to clone the purified PCR product, ligation was performed using StrataClone PCR Cloning Kit (Stratagene, USA). Ligated plasmids were then transformed into highly competent *Escherichia coli* and the transformants were selected according to the standard blue-white screening procedure (Sambrook and Russell, 2001). White colonies were isolated and cultured in Luria-Bertani broth containing 100µg ml^{-1} ampicillin. Colony PCR was conducted with primers P27F and P1492R for the selection of recombinant clones. The plasmids with 16S rDNA insert were isolated using a Nucleospin Plasmid DNA purification kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. The presence of 1.5 Kb PCR product was confirmed by PCR of purified plasmid using primers P27F and P1492R as described earlier.

DNA sequencing and phylogenetic study

Nucleotide sequencing of both strands of cloned DNA was performed by automated DNA sequencing services provided by Xcelris Labs Ltd, Ahmedabad, India. Universal primers (T7: 5′-CATTATGCTGAGTGAGTGATATCC - 3′ and T3: 5′-AATTAACCCTCACTAAAGGGAA- 3′) were used for sequencing. The sequences obtained were analyzed using the BioEdit program and aligned using ClustalW program (Larkin et al., 2007). A phylogenetic tree was constructed with 16S rDNA sequences of our cultures along with sequences of standard cultures retrieved from Genbank database, by neighbor-joining method using Mega version 5.05 (Tamura et al., 2011).

Sugar utilization by Gal$^+$ isolates in broth cultures

J8 broth medium was prepared as described by Thomas and Crow (1984). To the sterile J8 medium 1% (w/v) galactose was added from 20% (w/v) stock solution which was sterilized with a 0.22µm pore size membrane filter (Milipore Corp, Bedford, Mass). The inoculum was prepared as reported by Somkuti and Steinberg (1979). All cell suspensions were incubated at 42°C and the samples were withdrawn at 2, 4, 6 and 8 h interval for analysis of cell numbers, pH and sugar content in the medium. The pH of the media was estimated using pH meter (Thermo Electron, San Jose, CA, USA). Cell numbers were determined by pour plating on M17 agar and the plates were incubated at 42°C for 24 h. Residual lactose and galactose in culture supernatant fluids were determined using a lactose and galactose quantification kit (Megazyme, Ireland).

Analysis of sugar utilization in milk

The cultures were grown in sterile reconstituted 10% (w/v) Non Fat Dry Milk (NFDM) for 8 h at 42°C. Samples were aseptically withdrawn at 2, 4, 6 and 8 h and analyzed for microbial count, titratable acidity and sugar content in milk. Enumeration of *S. thermophilus* from milk was carried out by pour plating on M17 agar followed by incubation at 42°C for 24 h. Titratable acidity was determined by the procedure described in IS: 1479, Part I, (BIS, 1960). Acidity was determined by titration with 0.1N NaOH using phenolphthalein as an indicator. Residual lactose and galactose in culture supernatant fluids were determined using lactose and galactose quantification kit (Megazyme, Ireland).

Stability of galactose positive *S. thermophilus* cultures

Active 14 h old cultures were inoculated in J8 broth containing 0.5% (w/v) galactose and sterile skim milk (10% NFDM). Cultures were then incubated at 42°C and sub-culturing was done consecutively for 10 times for every 12 h into J8 broth and skim milk. After each sub culturing, population of the Gal$^+$ strains were analysed by plating on Elliker agar with 0.5% galactose to check the stability of strains.

Low browning Mozzarella cheese

Preparation of low browning Mozzarella cheese

Mozzarella cheese was prepared according to the method described by Mukherjee and Hutkins (1994). The cheese was prepared from pasteurized (62.8°C for 30 min) milk procured from the experimental dairy plant of the National Dairy Research Institute, Karnal, Haryana, India. The combinations of starters used for mozzarella cheese preparation were as follows: Gal$^+$ *S. thermophilus* NCDC 659, NCDC 660 and NCDC 661 were paired individually with Gal$^-$ *L. bulgaricus* NCDC 04 and Gal$^+$ *L. helveticus* NCDC 292 respectively. It was compared with Gal$^-$ *S. thermophilus* NCDC 218 by pairing with Gal$^-$ *L. bulgaricus* NCDC 04 and Gal$^+$ *L. helveticus* NCDC 292 respectively and it served as control. Cheese making trials were performed in duplicates for each combination and the mean of the all values were used for final analysis. Mixed milk was standardized to a 3.0% fat level and pasteurized. It was cooled to 32°C and it was inoculated with 2% of a combination starter (1:1). Milk was allowed to ripen for 1 h at 32°C. Microbial rennet (Fromase) was added at the rate of 0.0016g/100ml and the curd was cut after 30 to 35 min. Heating of curd was done at 41^{o}C for 30 min, with periodic stirring. Whey was drained at curd pH of 5.9 and curd was cheddared till pH 5.2. The curd was milled and molded in fresh water, maintained at 82°C, until curd balls were smooth and elastic. This required approximately 5 min for each sample. Molded curd balls were kept in ice water to make the curd firm. These balls were then placed in 20% (w/w) brine solution for an hour at 22°C. The cheese balls were wrapped in aluminium foil and stored under ice in a refrigerator.

Analysis of cheese

Samples were taken at various stages of the cheese making process including samples from curd at cutting, draining and cheddaring. Samples were frozen at -20°C in whirlpak bags for the analysis of sugars. Browning was carried out immediately after cheese making. The surface color was measured according to the method described by Mukherjee and Hutkins (1994). The surface color of Mozzarella cheese was measured using a "Colorflex" colorimeter (Hunter Associates Laboratory, Inc, Reston, VA, USA). Sugar analysis was carried out according to the procedure described in the lactose and galactose quantification kit (Megazyme, Ireland).

Statistical analysis

Descriptive statistics (mean±standard deviation [SD]) were used and analysis of variance with post hoc test (Bonferroni) at 95% significance level ($p<0.05$) was done to compare the different isolates with reference culture. The data was statistically analyzed with the application of SYSTAT 6.0.1 (Statistical Software Package, SPSS, USA).

Results

Confirmation of *S. thermophilus* for galactose fermentation

All six isolates, except the standard culture NCDC 218, exhibited a distinct yellow zone surrounding the colony confirming acid production from galactose in Elliker medium.

Phenotypic identification

All six isolates and reference strain were found to be Gram positive cocci in chains. All were catalase negative and they exhibited growth at 45°C and failed to grow at 10°C in the presence of 6.5% NaCl and did not hydrolyze bile esculin except NCDC 572 strain.

These isolates were further screened on the basis of their ability to ferment different sugars by API 50CHL and API 20 Strep test kits. All six isolates fermented lactose, glucose, sucrose and galactose. However, the complete sugar fermentation profile of each isolate was different from one another. D-Ribose was fermented by NCDC 659 and NCDC 660 and they fermented xylose at a slow rate, whereas, other strains did not ferment xylose. Mannose was fermented by NCDC 527, NCDC 572 and NCDC 547, but NCDC 659, NCDC 660 and NCDC 661 were found to be negative. According to API 20 Strep results, isolates NCDC 659, NCDC 660, NCDC 661 and NCDC 527 were identified as *S. thermophilus*, whereas isolates NCDC 547 and NCDC 572 were identified as *S. infantarius* and *S. macedonicus*, respectively.

Genotypic characterization of streptococcal isolates Species specific PCR

Species-specific PCR generated a specific amplification product of 968bp for the four isolates i.e., NCDC 527, 659, 660 and 661 along with the reference strain NCDC 218 confirming their identity as *S. thermophilus*, which was also inferred from the API 20 Strep results. No PCR products were obtained for isolates NCDC 547 and 572 and were identified as *S. infantarius* and *S. macedonicus*, respectively by API20Strep strip.

Cloning and sequencing of 16S rDNA gene from streptococcal isolates

Amplified PCR product of 1500 bp of each streptococcal strain was cloned and sequenced into a Strataclone PCR cloning kit for the sequencing of 16S rDNA gene. The six 16s rDNA nucleotide sequences obtained in this study was submitted to the GenBank database under accession number JN561295 to JN561300. In sequence similarity search using BLAST analysis of NCBI, the four isolates i.e. NCDC 527, 659, 660 and 661 showed 98% similarity to *S. thermophilus* JIM8232 (FR875178.1), *S. thermophilus* LMD9 (CP000419.1), *S. thermophilus* ND03 (CP0002340.1), *S. thermophilus* ATCC 19258 (AY188354.1), *S. thermophilus* LMG18311 (CP000023.1), *S. thermophilus* CNRZ1066 (CP000024.1) and *S. thermophilus* ATCC19984 (AY68738.1). Whereas, the isolate NCDC 547 showed a 99% similarity with *S. infantarius* 908 (EU163504.1) and *S. lutetiensis* (EU163503.1) and isolate NCDC 572 showed a 99% similarity index to *S. macedonicus* FM1830 (HQ7221250) and *S. macedonicus* strain 905 (EU163501.1). Thus the two Gal^+ isolates NCDC 547 and 572 were identified as dissimilar species other than *S. thermophilus*. This was also noticed with the API Strep 20 strip and species-specific PCR where no amplification was obtained for these two isolates.

Multiple sequence alignment was performed using ClustalWprogram (Larkin et al., 2007) for the 16S rRNA gene sequences of isolates along with *S. thermophilus* LMG18311 and *S. thermophilus* ATCC 19987 and other closely related *Streptococcus* spp. The phylogenetic tree was constructed by the UPGMA method using Mega 5.05 program. It formed seven clusters at a 98% similarity level: cluster 1 consisted of 3 isolates (NCDC 659, 660 and 661) along with reference strains *S. thermophilus* LMG18311 and *S. thermophilus* ATCC 19987. NCDC 527 fell into a separate group in cluster three. Whereas, NCDC 572 and *S. macedonicus* fell in cluster four. NCDC 547 and *S. infantarius* were grouped in cluster 5 (Fig. 1).

In the present study, all four *S. thermophilus* isolates were identified by both conventional and molecular methods. Results obtained from the 16S rRNA gene also confirmed the results of API20 Strep. The isolate NCDC 527 was identified as *S. thermophilus* by species-specific PCR but it did not grow in medium containing 0.5% galactose as the only sugar. Thus, only three galactose fermenting *S. thermophilus* isolates (NCDC 659, 660 and 661) were selected for further studies.

Sugar utilization of Gal^+ isolates in J8 broth containing 1% galactose

The amount of galactose utilized and pH reduction in J8 broth containing 1% (v/w) galactose was estimated for three Gal^+ *S. thermophilus* isolates at 2 h intervals up to 8 h at 42°C. Galactose utilization by all three isolates were significantly different ($p<0.05$) at 2, 4, 6 and 8 h interval. At 2 h, there was no significant difference between the isolates in galactose utilization. The Gal^+ isolate, NCDC 659 grew actively in galactose at 2, 4, 6 and 8 h respectively which was followed by NCDC 661 and NCDC 660 respectively (Table 1).

Correspondingly, a significant reduction in pH was observed from 2 h to 8 h in all the isolates, with a significant utilization of galactose. Among the 4 different time intervals, significant difference ($p<0.05$) between the isolates was observed at 4, 6 and 8 h than at 2 h ($p<0.05$). The pH was reduced from 7 to 4.92 by NCDC 659, 7 to 6.2 in NCDC 661 and 7 to 6.69 in NCDC 660. The viable count increased from 5.98±0.04log $CFUml^{-1}$ to 9.02±0.03 log $CFUml^{-1}$ in NCDC 659, 6.01±0.04 log $CFUml^{-1}$ to 8.96±0.01 log $CFUml^{-1}$ in NCDC 661 and 5.98±0.04 log $CFUml^{-1}$ to 8.03±0.04 log $CFUml^{-1}$ in NCDC 660.

Sugar utilization in milk

The galactose fermenting isolates were incubated in milk for 8 h and titratable acidity, sugar content and viable count of *S. thermophilus* were analysed at 2 h intervals (Table 2). The galactose release was lower in NCDC 659 (0.24±0.04), followed by NCDC 661 (0.34±0.03) and NCDC 669 (0.39±0.009) after 8 h when compared with the Gal^- control, NCDC 218 which expelled higher amount of galactose (0.68±0.001). Similarly, pH reduction and an increase in titratable acidity showed a significant ($P<0.05$) difference at 4, 6 and 8 h of incubation.

Stability of Gal^+ *S. thermophilus* isolates during sub-culturing in lactose excess media

All cultures were found to be more stable when sub cultured in broth medium than in milk. NCDC 661, 659 and 660 showed good stability in broth up to 10 subcultures i.e., 85%, 75% and 72%, respectively (Table 3).

Preparation of Mozzarella cheese using galactose$^+$ *S. thermophilus* isolates

Mozzarella cheese was prepared using combination of Gal^+ starter cultures, in order to evaluate its suitability as cheese starters as well as its impact on browning tendency which occurred during pizza baking was also evaluated.

Galactose accumulation in Mozzarella cheese

Cheese making trials were done using all combinations. Analysis was done for the sugar content during different stages of cheese manufacture (Table 4). In this study, the lactose concentration ranged from 2.65 to 3.17% at cutting and it reduced rapidly from 0.26% to 1.08% after cheddaring. Mozarella cheese possessed less concentration of galactose, when prepared with Gal^+ cultures. The lowest galactose accumulation occurred in Gal^+ *L. helveticus* NCDC 292 pairing with Gal+ *S. thermophilus* NCDC 659 (0.15%) followed by Gal+ NCDC 661 (0.30%), NCDC 660 (0.33%). The galactose accumulation in Gal^- *L. bulgaricus* NCDC 04 pairing with NCDC 659 was 0.36% followed by NCDC 660 (0.53%), NCDC 661 (0.59%) and standard Gal^- *S. thermophilus* NCDC 218 (0.88%). The combination of Gal^+ *S. thermophilus* NCDC 659 with Gal^+ *L. helveticus* NCDC 292 resulted in the lowest concentration of galactose which was significantly lower than the control Gal^- *S. thermophilus* NCDC 218 when paired with both Gal+ *L. helveticus* NCDC 292 and Gal- *L. bulgaricus* NCDC 04.

Browning of Mozzarella cheese

Browning was measured by baking through convectional heating. Cheese samples were cooked at 250°C for 2 min. Then cooled samples were analyzed with a Hunterlab colorimeter for three indices using L*, a*, b* scales. L* value indicates color change differences from black to white (0-100) and was used to evaluate differences in the cooking color. In the present study, L* ranged from 66.68±0.4 to 76.09±0.37 confirming that Mozzarella cheese had a relatively dark color compared with the standard white color (L*: 100). Among the isolates, cheese prepared using Gal^+ *S. thermophilus* NCDC 659 showed better L*(76.09±0.37) followed by NCDC 661 (73.43±0.15), while Gal^- *S. thermophilus* NCDC 218 showed least L* value 66.68±0.4. In contrast, differences in a* values (red to green) became more positive indicating a more intense red color. In the present work, the control sample using NCDC 218 showed the highest red value (-0.34±0.58) indicating that maximum browning took place in control, whereas Gal^+ NCDC 659, 660 and 661 had less a* value indicating less browning. This may be associative with their high L* value, although, the difference in values of the various samples are not statistically ($p<0.05$) significant (Table 5). The b* value indicates color change from yellow to blue. The b* value was least affected by cooking, and most values did not change significantly between samples. Hence from the present study it is clear that the combination of Gal^+ *S. thermophilus* NCDC 659 and Gal^+ *L. helveticus* NCDC 292 exhibits higher values of L* and b* and the lower value for a*, thereby revealing its least potential for browning. The study also clearly emphasizes that Gal^+ strains play a critical role in controlling release of galactose and thereby browning.

Fig 1. Dendrogram showing phylogenetic relationship of *Streptococcus thermophilus* reference strains retrieved from GenBank with the galactose positive streptococcal strains isolated from plant and milk samples based on 16S rRNA gene sequencing.

Fig.1

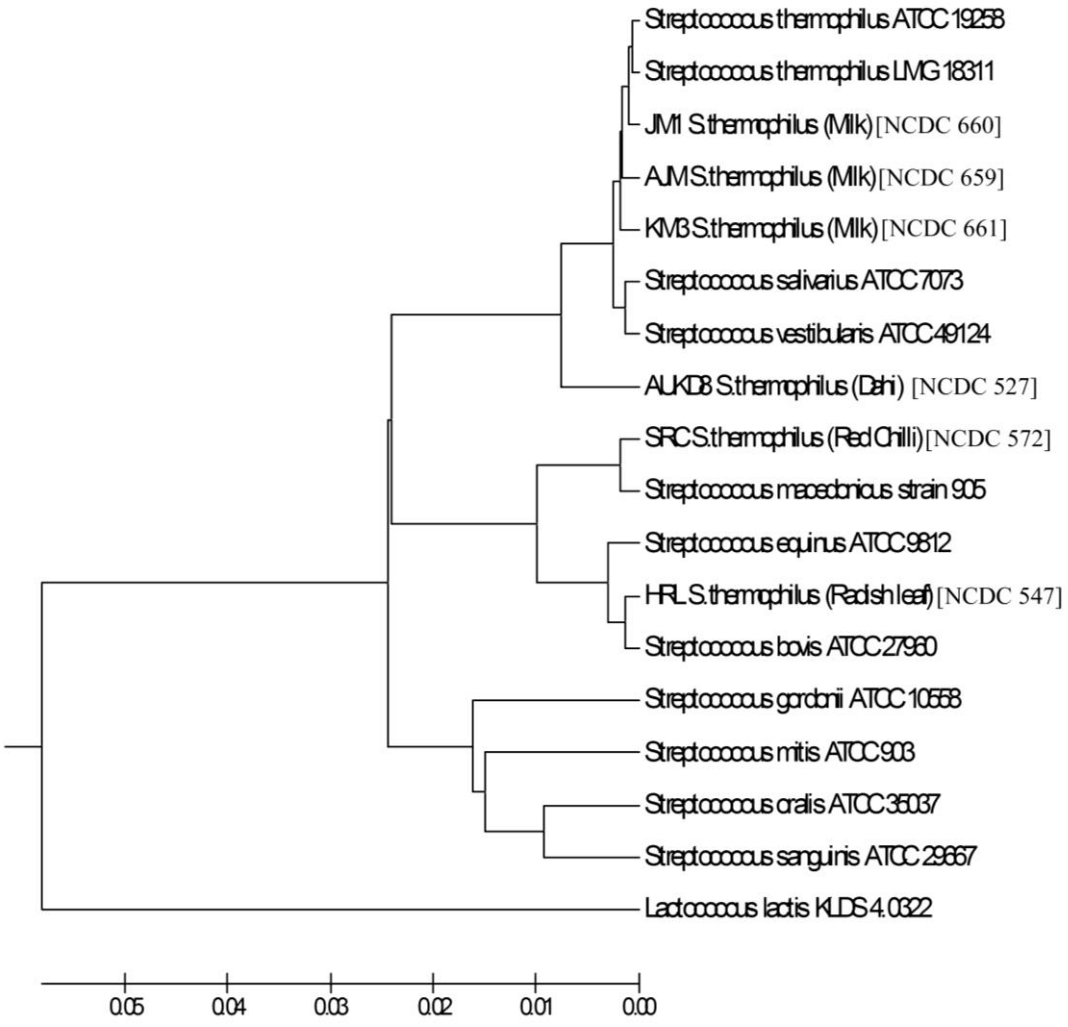

Table 1. Estimation of galactose utilization in J8 broth containing 1% galactose at regular intervals by gal$^+$ isolates of *S.thermophilus*

S.No.	ISOLATES	PARAMETERS	INCUBATION (h)				
			0 h	2 h	4 h	6 h	8 h
1	NCDC 659	GALACTOSE (%)	0.0	0.20±0.001	0.32±0.01	0.41±0.003	0.44±0.014
		GALACTOSE (gL^{-1})	0.0	2.0	3.2	4.1	4.4
		pH	7.0±0.00	6.81±0.001	6.50±0.01	5.10±0.05	4.97±0.06
		LOG $CFUm^{-1}$	5.98±0.04	6.34±0.07	6.93±0.11	7.71±0.10	9.02±0.03
2	NCDC 661	GALACTOSE (%)	0.0	0.18±0.006	0.30±0.003	0.31±0.01	0.36±0.02
		GALACTOSE (gL^{-1})	0.0	1.8	3.0	3.1	3.6
		pH	7.0±0.00	6.93±0.02	6.9±0.02	6.45±0.14	6.2±0.12
		LOG $CFUm^{-1}$	6.01±0.04	6.16±0.01	6.97±0.29	8.01±0.44	8.96±0.04
3	NCDC 660	GALACTOSE (%)	0.0	0.21±0.03	0.22±0.01	0.24±0.06	0.29±0.002
		GALACTOSE (gL^{-1})	0.0	2.1	2.2	2.4	2.9
		pH	7.0±0.00	6.90±0.03	6.85±0.01	6.73±0.03	6.69±0.06
		LOG $CFUm^{-1}$	5.98±0.04	6.13±0.27	6.93±0.07	7.77±0.06	8.03±0.04

Values are presented as Mean±SD, of three individual experiments (n=3).

Table 2. Fermentation of milk with the Gal$^+$ isolates at regular intervals of time

S.No.	ISOLATES	PARAMETERS	INCUBATION (h)			
			0 h	4 h	6 h	8 h
1	NCDC 659	LACTOSE (%)	4.24±0.18	3.38±0.07	3.04±0.04	2.81±0.03
		GALACTOSE (%)	0.03±0.008	0.09±0.02	0.16±0.005	0.24±0.04
		pH	6.27±0.01	5.63±0.02	5.19±0.05	4.75±0.02
		ACIDITY (%)	0.14±0.001	0.23±0.007	0.38±0.002	0.40±0.02
		LOG $CFUm^{-1}$	6.20±0.05	8.84±0.03	9.27±0.02	9.77±0.02
2	NCDC 660	LACTOSE (%)	4.24±0.18	3.49±0.11	3.17±0.04	2.87±0.06
		GALACTOSE (%)	0.03±0.008	0.25±0.01	0.30±0.01	0.39±0.009
		pH	6.27±0.01	5.65±0.02	5.17±0.01	4.72±0.01
		ACIDITY (%)	0.14±0.001	0.22±0.008	0.36±0.01	0.39±0.01
		LOG $CFUm^{-1}$	6.40±0.05	8.87±0.04	9.07±0.05	9.93±0.04
3	NCDC 661	LACTOSE (%)	4.24±0.18	3.85±0.06	3.46±0.05	3.27±0.10
		GALACTOSE (%)	0.03±0.008	0.11±0.05	0.2±0.02	0.34±0.03
		pH	6.27±0.01	5.7±0.02	5.23±0.02	4.74±0.02
		ACIDITY (%)	0.14±0.001	0.22±0.004	0.31±0.002	0.43±0.02
		LOG $CFUm^{-1}$	6.00±0.05	8.17±0.01	8.77±0.04	8.99±0.02
4	NCDC 218	LACTOSE (%)	4.24±0.18	3.63±0.02	3.05±0.02	2.35±0.12
		GALACTOSE (%)	0.02±0.006	0.32±0.02	0.44±0.02	0.68±0.001
		pH	6.50±0.03	6.08±0.04	5.35±0.02	4.93±0.01
		ACIDITY (%)	0.15±0.003	0.18±0.02	0.38±0.003	0.39±0.001

	LOG $CFUm^{-1}$	6.41±0.11	7.77±0.03	8.28±0.16	8.62±0.06

Table 3. Stability of S.thermophilus Gal^+ isolates sub cultured in lactose-excess media (Milk and J8 broth containing 5% lactose)

Isolates	Culture media	Percentage (%) of Gal^+ cells in population after specified number of subcultures			
		0	5	7	10
NCDC 659	Broth	99	96	88.75	75
	Milk	99	69	32.35	-
NCDC 660	Broth	99	84	81	72
	Milk	97	84	70	52
NCDC 661	Broth	99	98	93.5	85
	Milk	97	82	76	69

Table 4. Lactose and galactose concentration in fresh Mozzarella cheese (g/100g) prepared using different combination of cultures

S.No.	CULTURE	CUTTING		DRAINING		CHEDDARING	
		LACTOSE (%)	GALACTOSE (%)	LACTOSE (%)	GALACTOSE (%)	LACTOSE (%)	GALACTOSE (%)
	Gal^+ *S.thermophilus* + Gal^- *L. delbrueckii ssp. bulgaricus* NCDC04						
1	NCDC 659	$3.17±0.008^{a}$	$0.08±0.01^{a}$	$1.93±0.03^{a}$	$0.21±0.02^{A}$	$0.49±0.01^{afh}$	$0.36±0.03^{b}$
	NCDC 660	$2.74±0.04^{ab}$	$0.19±0.02^{a}$	$1.77±0.03^{ab}$	$0.34±0.03^{A}$	$0.43±0.03^{bf}$	$0.53±0.01^{b}$
	NCDC 661	$2.67±0.06^{ab}$	$0.12±0.002^{a}$	$1.21±0.07^{b}$	$0.34±0.01^{A}$	$0.24±0.11^{bc}$	$0.59±0.01^{b}$
	NCDC 218	$3.09±0.12^{a}$	$0.20±0.004^{a}$	$1.44±0.07^{ab}$	$0.55±0.001^{A}$	$0.55±0.05^{bd}$	0.88±0.003+
	Gal^+ *S.thermophilus* + Gal^+ *Lactobacillus helvetiucs* NCDC 292						
2	NCDC 659	$2.87±0.12^{ab}$	$0.05±0.008^{a}$	$1.32±0.03^{ab}$	$0.107±0.01^{A}$	$0.26±0.01^{ce}$	$0.15±0.01^{b}$
	NCDC 660	$2.65±0.08^{ab}$	$0.07±0.01^{a}$	$1.67±0.04^{ab}$	$0.21±0.007^{A}$	$0.39±0.009^{dfh}$	$0.33±0.06^{b}$
	NCDC 661	$2.75±0.07^{b}$	$0.05±0.002^{a}$	$1.56±0.05^{ab}$	$0.18±0.01^{A}$	$0.26±0.04b^{gh}$	$0.30±0.01^{b}$
	NCDC 218	$2.94±0.02^{ab}$	$0.15±0.03^{a}$	$1.98±0.07^{a}$	$0.29±0.006^{A}$	$0.39±0.04^{h}$	$0.49±0.002^{b}$

Values are presented as Mean±SD of two individual experiments (n=2)

Table 5. Hunterlab Index of Mozzarella cheese for different cultures

S.No.	CULTURES	L*(light to darkness)	b*(Yellow and blue)	a*(red to green)
1	NCDC 659+NCDC 04	$73.2±0.17^{a}$	$14.47±0.17^{a}$	$-3.84±0.68^{ab}$
2	NCDC 660+NCDC 04	$69.4±0.23^{b}$	$16.61±0.40^{bce}$	$-0.45±0.47^{bc}$
3	NCDC 661+NCDC 04	$72.63±0.11^{a}$	$15.88±0.05^{ab}$	$-1.62±0.08^{bc}$
4	NCDC 218+NCDC 04	$66.68±0.14^{d}$	$18.73±0.13^{d}$	$-1.21±0.01^{bc}$
5	NCDC 218+NCDC 292	$69.04±0.35^{be}$	$17.8±0.3^{e}$	$-0.34±0.58^{c}$
6	NCDC 660+NCDC 292	$72.26±0.24^{a}$	$14.90±0.24^{a}$	$-2.92±0.90^{bc}$
7	NCDC 659+NCDC 292	$76.09±0.37^{c}$	$14.41±0.42^{a}$	$-2.53±1.16^{bc}$
8	NCDC 661+NCDC 292	$73.43±0.15^{a}$	$17.64±0.35^{c}$	$-0.91±0.51^{bc}$

Values are presented as Mean±SD of three individual experiements (n=3). a,b,c,d,e - Mean values in the same column (corresponding to the same parameter) not followed by a common letter differ significantly ($p < 0.05$)

Discussion

S. thermophilus is widely used in the manufacture of yoghurt and cheese. In addition, some specific strains also have the potential to improve human health as probiotics. Generally, *S.thermophilus* strains are galactose negative. Accumulation of galactose in dairy products may lead to several undesirable effects. However, galactose positive strains of *S. thermophilus* may be valuable for the dairy industry to prevent problems caused by the accumulation of galactose in fermented milk products (Mukherjee and Hutkins 1994). In view of this, the study was carried out to screen galactose positive *S. thermophilus* strains obtained from plant and milk samples from different geographical regions of India.

Among the six streptococcal isolates examined, all isolates except the reference strain NCDC 218 exhibited a distinct yellow zone around the colony due to the acid production from galactose present in the Elliker-agar. All six Gal^+ isolates, including the reference strain, were examined for various physiological and biochemical characteristics for phenetic identification. Four out of the six isolates showed typical phenotypic features of *S. thermophilus*. Mukherjee and Hutkins (1994) and De Vin et al., (2005) isolated Gal^+ *S. thermophilus* from cheese and yogurt samples.

The ability to ferment various sugars is a major biochemical characteristic for the differentiation of *S. thermophilus* from other closely related species. *S. thermophilus* ferments lactose, glucose, fructose, mannose and sucrose (Holt, 1986) however, fermentation of sucrose, galactose and maltose are strain dependent property (Robinson et al., 2003). All the isolates were subjected to a sugar fermentation test along with reference strains using the API50CHL system. Altogether, from six isolates, only four showed typical sugar fermentation profile and other phenotypic reactions considered to be characteristic of *S. thermophilus*. The remaining 2 isolates were identified as *S. infantarius* and *S. macedonicus*, respectively. Although laboratories rely on phenotypic characterization as their primary method of identifying strains, but phenotypic tests may have some disadvantages as all strains within a given species may exhibit a common characteristic, the test result relies on individual interpretation and expertise, and small alterations in the execution of an assay may give false test results (Poyart et al., 1998; Bosshard et al., 2004). Hence, several workers report that conventional biochemical identification of *S. thermophilus* is not sufficient, as it introduces bias related with the ability of growth of the microorganisms under specific laboratory conditions. Hence the isolates were confirmed by species-specific PCR.

Many researchers suggested that species-specific PCR using *lacZ* gene is a rapid and accurate method for identification of *S. thermophilus* isolates (Andrighetto et al., 2002; Fortina et al., 2003; Marino et al., 2003; Giraffa et al., 2008; Erkus et al., 2013). The genotypic characterization by species-specific *lacZ* PCR primers has been used for the differentiation of *S. thermophilus* strains from other *Streptococcus* spp. and other lactic bacteria. In this study, out of six isolates, only four were confirmed as *S. thermophilus* by species-specific PCR and two isolates failed to produce products using *lacZ* primers.

In order to verify the taxonomic identity of the strains, the V1-V9 (1458bp) variable region of 16S rRNA were sequenced and compared to known sequences in databases. The results of the phylogenetic analysis based on the sequencing data was in agreement with the results obtained from API 50 CHL, API 20 Strep and species-specific PCR. The phylogenetic tree constructed from the obtained 16S rRNA gene sequence data with other standard culture gene sequences of *S. thermophilus*, were in agreement with the results obtained by Mora et al., (2003) and Botina et al., (2007). They reported that the nucleotide sequences of ribosomal genes are widely used for taxonomic identification of bacteria. They did comparative analysis of the nucleotide sequences of 16s rRNA gene of 72 *S. thermophilus* strains with the reference strain ATCC19258, which showed 100% homology. They suggested that it is preferable to use 16S rRNA gene sequencing data for identification of closely related lactic acid bacteria isolated from natural sources.

Three naturally isolated strains viz., NCDC 659, 660 and 661 were able to ferment and utilize galactose for growth. The Gal^+ isolate NCDC 659 grew actively in galactose which is followed by NCDC 661 and 660. Similar results were obtained by (Amoroso et al., 1989) as they reported that the concentration of galactose in LAPT-gal medium (1% galactose) decreased from 8.8g L^{-1}, 7.0g L^{-1}, 6.7g L^{-1} and 6.5g L^{-1} after 2, 4, 6 and 8 h of incubation with *S. thermophilus*. Moreover, the galactose release in skim milk at regular intervals increased with time and it was lower in NCDC 659 than NCDC 660 and 661. Hutkins et al., (1985) also reported a similar finding that the Gal^+ strain (KK1, KK2, KK3 and KK5) released less galactose (0.05%) than the Gal^- KK4

strain (0.06-0.22%) incubated in skim milk. Robitaille et al., (2009) reported that the Gal^+ EPS producing recombinant *S. thermophilus* RD-534-S1 released galactose 4.3 g kg^{-1} similar to the parental strain RD-534 4.4 g kg^{-1} after a 12 h fermentation of reconstituted skim milk at 40°C. However, lactic acid/galactose molar ratio was more in the recombinant strain than the parental strain through utilization of galactose for metabolic process (Robitaille et al., 2007; Vaillancourt et al., 2004).

Most strains of *S. thermophilus* are galactose negative. These cultures are traditionally maintained in milk where lactose is in excess and pH becomes the growth limiting factor (Thomas and Crow, 1984). They also reported that galactose positive isolate (MC) was more stable in broth (98%) when compared with sub-culturing in milk (34%). Thus, proper maintenance of Gal^+ cultures is needed since they are unstable when maintained in milk, reverting to the galactose negative phenotype. Master stocks of Gal^+ cultures should be maintained in media with galactose as the only sugar and bulk cultures may be prepared from master stocks (van den Bogaard et al., 2004).

Exploration of Gal^+ *S. thermophilus* isolates as a starter for the cheese making process is one of the stratagies to reduce the browning of the Mozzrella cheese. Johnson and Olson (1985) suggested that Mozzrella cheese made with galactose fermenting *S. thermophilus* has the capacity to reduce browning. In order to examine the impact of Gal^+ *S. thermophilus* on the dairy products, Mozzarella cheese was prepared.

Cheese making trials were done using all 8 culture combinations. Analysis was done for sugar content during different stages of cheese manufacture (Table 5). In this study, the lactose concentration ranged from 2.65 to 3.17% at cutting and it reduced rapidly (0.26% to 1.08%) after cheddaring. In the present study, Gal^+ *S. thermophilus* NCDC 659 paired with Gal^+ *L. helveticus* exhibited the lowest concentration of galactose than Gal^+ *S. thermophilus* paired with Gal^- *L. bulgaricus* NCDC 04. Similarly, Johnson and Olson (1985) reported that Mozzarella cheese made with Gal^+ *S. thermophilus* and Gal^+ *L. bulgaricus* contained as low as 0.1% galactose (after 28 days of storage) as compared to 0.78% as in the case of Mozzarella prepared with Gal^- *S. thermophilus* and Gal^- *L. delbrueckii* subsp *bulgaricus*. Baskaran and Sivakumar (2003) reported that the galactose concentrations in Mozzarella cheese made with Gal^+ *S. thermophilus* and *L. helveticus* was less (0.3%) than cheese made with Gal^+*S. thermophilus* and *L. bulgaricus* (0.7%) and Gal^- *S. thermophilus* and *L. bulgaricus* (0.83%). Mukherjee and Hutkins (1994) reported that Mozzarella cheese made with galactose non-releasing strain (KK-1, KK-2, KK-3) contained less galactose (<0.1%) than galactose releasing strain KK-4 (0.2%). Five times more galactose (0.6%) contributed to the high browning of cheese compared to low browning cheese (0.13%) (Matzdorf et al., 1994). Robitaille et al., (2007) reported that Mozzarella cheese prepared with the recombinant strain MR-AAC accumulated less galactose (7.3g/kg) than parental strain MR-AC (9.0g/kg).

Conclusion

The availability of Gal^+ strains of *S. thermophilus* for the manufacture of Swiss, Cheddar, Mozzarella and other milk products requiring thermophilic starter culture would be of considerable value to the dairy industry. In this study, using Gal^+ strain of *S. thermophilus*, the presence of residual amount of galactose in broth and milk after 8 h incubation indicated that the Gal^+ strains were unable to fully utilize the galactose generated from lactose metabolism. On the basis of the results, the Gal^+ strains of *S.thermophilus* used in the study were paired with Gal^+ *L. helveticus* and Gal^- *L. bulgaricus* for manufacture of Mozzarella cheese in order to utilize galactose more efficiently than Gal^- *S. thermophilus* used in traditional Mozzarella cheese. In conclusion, this study shows that Gal^+ isolates obtained in this study can be successfully paired with Gal^+ *L. helveticus* to prepare defined strain starters for low browning Mozzarella. The major limitation of Gal^+ cultures is their unstability in milk and they tend to revert to Gal^- phenotype, which has been overcome by the Gal+ cultures obtained in this study.

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