The Pharma Innovation Journal 2022; SP-11(7): 4539-4544

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(7): 4539-4544

www.thepharmajournal.com
Received: 19-04-2022
Accepted: 22-05-2022

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Preparation of fermented oat drink using lactococcal isolates

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Abstract
The number of people consuming dairy products is not increasing due to intolerance or allergies to food components. Consumers are increasingly searching for alternative, nondairy beverages which have an advantageous effect on the body and which stimulate gut microflora. The oat drink is very important for health. Different *dahi*, vegetable and fermented rice samples were collected from local market and households of Chhattisgarh State. The samples were used for isolation of pure cultures (60) by streaking on M-17 medium. All the isolates were found to be Gram-positive cocci existing in pairs and small to medium chains, catalase negative. Sugar fermentation profile of the isolates showed a carbohydrate utilization pattern similar to reported pattern of genus *Lactococcus*. Based on morphological and biochemical identification all the 60 isolates were presumptively considered as belonging to genus *Lactococcus*.

Keywords: Fermentation, lactococci, oat drink, *dahi* samples, carbohydrate utilization

Introduction
Lactic acid bacteria are an order of gram-positive, low-GC, acid-tolerant, generally non-sporulating, non-respiring, either rod-shaped (bacilli) or spherical (cocci) bacteria that share common metabolic and physiological characteristics. These bacteria, usually found in decomposing plants and milk products, produce lactic acid as the major metabolic end product of carbohydrate fermentation. Lactic acid bacteria either undergo homo or hetero fermentation to produce their energy for cell functions and produce biomass. The LAB contain the genera *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Streptococcus*, *Lactococcus*, *Pediococcus* etc., mostly associated with dairy environment. Lactococci are used extensively in food fermentation, which represent about 20% of the total economic value of fermented foods produced throughout the world. Lactococci are coccoid Gram-positive, catalase-negative, non-motile and facultative anaerobic bacteria, with L-(+)-lactic acid as their predominant end product of glucose fermentation (Li *et al*., 2020) [12].
The common morphology of lactococci consists of 0.5 to 1-µm diameter spheres or ovoid cells that exist in pairs or chains. Cells of lactococci often elongate in the direction of the chain, which makes them difficult to differentiate from lactobacilli. Lactococcal cultures usually grow in the range 10-30 ºC, although some species may grow under temperatures as low as 7 ºC upon prolonged incubation for 10–14 days. Cultures typically grow in 4.0 % (w/v) NaCl; however, *Lac. Lactis* subsp. *cremoris* tolerates only 2.0 % NaCl, which is the only known exception. Lactococci grow best at near neutral pH values in media but cease to grow at about pH 4.5. Lactococci are homo-fermentative; when grown in milk, more than 95% of their end product is lactic acid (of the L isomer). Lactococci are typically used for the production dairy products. Within species *Lactococcus lactis*, two sub species *L. lactis subsp. lactis* and *cremoris* are the most widely being used for dairy fermentation. Lactococcal cultures play a key role for determining the quality of fermented dairy products with respect to shelf-life and sensory quality. A high degree of sequence similarity exists between Streptococcaceae, yet they can be found in a broad range of different environmental niches. Strains of *L. lactis* have been isolated from a range of sources including drain water and human vaginal samples (Gao *et al*., 2011; Kato *et al*., 2012) [4, 8]. Although not a common resident of the gastro intestinal tract (GIT), *L. lactis* is capable of surviving gut passage (Kimoto *et al*., 1999; Meyrand *et al*., 2013) [10, 14].

Materials and Methods

Isolation of Lactococci Cultures from Dairy and Vegetable Samples.

A total number of 40 dairy and 15 vegetable, and 5 fermented rice samples were collected from the rural and urban areas of the Chhattisgarh state. Dairy samples included *Dahi*, Butter milk, vegetable samples included Cucumber, Brinjal, Chilli, Tomato, Lady Finger, Cauliflower, Broccoli and Rice included fermented rice. Each sample was taken in a sterile container separately and placed in sterile sample containers during transportation to the laboratory, brought to the laboratory and stored in refrigerated conditions till further processing. Growth medium for isolation of Lactococci cultures, M-17 medium supplemented with β-di-sodium glycerophosphate was used as culture medium (Terzaghi and Sandine, 1975) [22]. Curd samples were prepared by mixing 10 ml aseptically weighed samples to 90 ml of sterile saline (0.85% sodium chloride). The vegetable samples were enriched by transferring to M-17 broth tubes and incubated at 30 ˚C for 24-48 h. Then enriched broth medium was then serially diluted in sterile saline tubes (1:10) and then pour plated using appropriate dilution. Before inoculation of sample, the pH of M-17 broth was adjusted to 6.5 ± 0.2. The enriched samples were streaked on the Petri plates containing Lactococci selection M-17 agar with the help of calibrated inoculating loop and incubated aerobically at 30 °C for 48 h and observed for the growth of colonies.

Identification of Lactococci Cultures

All the randomly selected colonies were tested for morphology and purity by microscopic examination. The isolates which showed typical morphology were further subjected to biochemical identification.

Morphological Characterization

The isolates were checked for purity and morphology by Negative straining and Gram staining. Thin smear of active MRS broth culture was prepared on glass slides. The smear was heat fixed and stained using Gram Stain kit (Himedia). After drying slides were observed under oil immersion objective (100X) for Gram's reaction, shape and arrangements of cells. Gram positive cocci in pairs/chains were further subjected to biochemical characterization.

Biochemical Characterization

An array of physiological and biochemical tests was performed as per standard methods (Mannu *et al.*, 2000) [13] to identify the morphologically selected isolates. The pure cultures were grown in M-17 broth at 30 ℃ for 24 h. The 24 h activated cultures were used as inoculums for the various physiological and biochemical tests employed to identify the isolated cultures.

Catalase Test

An aliquot of the 24 h M-17 broth cultures was transferred on to a clean glass slide and a few drops of 3 per cent hydrogen peroxide were poured over it and observed for effervescence. The presence of effervescence indicated positive for catalase test whereas no effervescence was taken as negative test.

Carbohydrate Fermentation

Tentative identification of isolates was done mainly on the basis of sugar fermentation pattern. The ability of the cultures to ferment and produce acid from various sugars was tested in Hi-Carbo Kit (Part-A) (Hi Media Laboratories Pvt. Ltd., Mumbai, India) which contained different sugar wells. The kit was having twelve immobilized carbohydrates in wells viz. Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose, L-Arabinose and Mannose. The sugar fermentation was carried out as per manufacturer's instructions. Briefly, the cultures were grown in 10 ml M-17 broth at 30 ºC for 24 h and cells were harvested by centrifugation in refrigerated centrifuge. The supernatant was discarded carefully and pellet was washed using sterilized saline. The washed cells were suspended in 5 ml of saline, a part of which was used for assessing O.D. using spectrophotometer. The prepared inoculum was inoculated @ 50 µl in each well of the kit and incubated at 30 ºC for 24-48 h. After stipulated incubation change in color of immobilized sugar was observed and noted down.

Growth in Presence of NaCl Concentration

The ability of isolates to grow in presence of different concentrations of NaCl was tested by inoculating in 5ml of M-17 broth tubes containing 4 per cent, 6.5 per cent NaCl and 9 per cent NaCl, respectively. The broth tubes were incubated at 30 °C for 48 h. Increase in turbidity of the broth medium was taken as positive test, whereas no increase in turbidity was taken as negative test.

Maintenance of Cultures

All the isolates were maintained in M-17 broth at 4 ºC and sub-cultured once in a week. A stock of the isolates was also preserved in 50per cent glycerol stock medium (Appendix) at -20 ºC. The cultures were also stabbed in M-17 medium tubes and kept at 4 ºC for long term storage. The cultures were activated prior to use by sub culturing twice in M-17 broth. Strain purity was checked regularly by microscopy, colony morphology, Gram's test and catalase activity.

Fermentation of Oat Drink

The process of extraction of oat drink from oats grain as explained in a study by Sangami and Radhaisri (2018) [19] was followed. Split oats grain was used for the milk extraction as it retains better yield than the whole grain or oat meal. Prior to soaking, the spilt oat grain was tempered in water boiled at 80 °C for 3 mins and drained. The tempered oats grain was then soaked in 150 ml of sterilized hot water for 12 h at room temperature, ground in a mixer grinder and the slurry filtered using a nut milk bag till the oats milk is completely extracted. Preparation of fermented oat drink using lactococci isolate and the concentration of Lactococci isolate (2-4%) to be used for fermentation of oat drink. Duration of fermentation to attain desirable colony counts of 10^8 CFU/mL M-17 agar. The best isolate was selected on the basis of final acidity and sensory evaluation.

Sensory Evaluation

Organoleptic evaluation of the fermented oats drink was performed with ten trained panelist in analyzing the colour, flavour, consistency, mouth feel, taste and overall acceptability based on a nine-point hedonic scale ranging from 1 (dislike extremely) to 9 (like extremely) (Wichchukit and Omahony, 2015) [21].

Titratable Acidity

Titratable acidity (as lactic acid content) and pH, oat sample was used for analysis. For the titratable acidity assay, 10.0 ml

of oat sample was titrated against 0.1 N NaOH using phenolphthalein as an indicator (pH 8.3). Titration was initiated by adding 2–3 drops of phenolphthalein to the sample, followed by drop wise addition of 0.1 N NaOH with continuous swirling. The appearance of a stable light pink color indicated the point of neutrality, i.e., the end point of the titration. The amount of NaOH used (titre) was recorded and titratable acidity was expressed by using the following equation: 1.0 ml of 0.1 N NaOH = 0.0090 g of lactic acid.

Results and Discussions

Isolation of Lactococci from Collected Samples

Collected dairy and vegetable samples were directly submitted to isolation of lactococcal strains. Vegetable samples were crushed in a pestle-mortor and mixed with appropriated volume of saline. Samples were serially diluted (10^1-10^6) in saline (0.85%) solution and then plated on M-17 agar by using streak plate method (Fig. 1). The plates were incubated at 30 ˚C for 48 h and then several colonies were picked randomly for identification of Lactococci. Single well separated colonies were randomly picked with a sterile toothpick and inoculated in M-17 broth and incubated at 30 ˚C for 24-48 h. A total 60 isolates of potential Lactococci were isolated in pure culture. These cultures were maintained by frequent sub-culturing in M-17 broth and preserved for long-term storage in glycerol stocks at -20 ˚C. All the isolates were subjected to poly-phasic identification procedure. The lactococci are usually adapted to nutrient rich environments due to their auxotrophic nature. Lactococci have been traditionally associated dairy products particularly fermented milk products such as cheese, dahi, buttermilk. Lactococcal isolates have been isolated from diverse niches by various workers, which include vegetables (Kelly *et al.* 1998; Salama *et al*. 1995; Ponce *et al*. 2008) [9, 18, 17], dahi, butter milk (Harun-ur-Rashid *et al*. 2007; Mitra *et al.* 2007) [6, 15]. Bettache and Fatma (2012) [1] studied 5 samples of traditional fermented milk butter collected from individual households of Algeria. Lactic acid bacteria dominated the microflora of these samples, especially the genera *Lactococcus, Leuconostoc and Lactobacillus.* Other groups identified included pyogenic streptococci and enterococci. The dominant *Lactococcus* species was *Lactococcus lactis* subsp. *lactis.*

Morphological Characterization

The isolated pure cultures of tentative lactococcal strains were examined for Negative staining and Gram reaction for morphological identification. Negative staining using nigrosine was used to visualize the cells against a dark background for clear morphological viewing (fig 2). The Gram Reaction of the isolates and morphological features are given in Table 1. All the tested strains were Gram positive and were cocci shaped mostly occurring in small-medium chains and some in pairs (Fig. 3). Lactococci is a genus of the group LAB and thus is Gram positive and occurs in cocci shape. Based on morphological examination of isolates they appear similar to lactococci but would require further confirmation using biochemical tests.

Table I: Cell morphology and catalase test of the isolated lactococci

Isolates No.	Gram Reaction	Shape and Arrangement	Catalase
1,5,6,12,15,25,27,33,57	+ve	Cocci in short chain	-ve
2,4,21,23,30,34,36,37,38,40,42,44,46,48,51,53,56	+ve	Cocci in pairs	-ve
7,11,13,14,22,24,32,35,39,41,43,45,47,55,58	+ve	Cocci in medium chain	-ve
3,8,19,52,54	+ve	Cocci in pair and short chain	-ve
16,18,60	+ve	Cocci in pair and medium chain	-ve
20,26,28,29,31	+ve	Cocci in pair and single	-ve
9,10,50,59	+ve	Cocci in medium and short chain	-ve

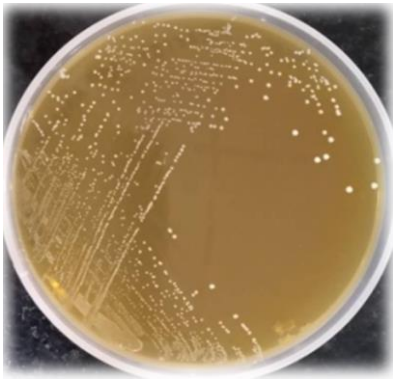

Fig 1: Streak plate of Sample

Catalase Test

The catalase test is performed on cultures to check their ability to express the enzyme catalase, which catalyzes the degradation of hydrogen peroxide to H_2O and O_2. The oxygen released in the reaction can be seen in the form of effervesce. This test is often used for identification of LAB as they lack the catalase enzyme. The test was carried out by placing a small volume of culture on a microscopic slide and then placing a drop of 3.0% H_2O_2. All the tested isolates did not show any effervescence when treated with 3.0% H_2O_2 which indicates that the isolates presumptively belong to the LAB group. The results of catalase test for the isolates are listed in table 1.

Growth in the Presence of 4.0, 6.5 and 9.0 % NaCl Concentration

M-17 broth tubes containing different concentration of NaCl (4%, 6.5% and 9%) were inoculated @ 1% inoculums of active culture and incubated at 30 ºC 24-48 h. The growth was assessed by increase in turbidity in the medium after 24-48 h.

Ability to grow at different NaCl concentration is a convenient test to differentiate and partially identify different cocci cells. The results of ability of isolates to grow at varying salt concentration are shown in table 2. All the 45 isolates were tolerant to 4.0% NaCl concentration and only 15 of them were able to tolerate a NaCl concentration of 6.5%. None of the 60 isolates was able to grow at 9.0 % salt concentration. Enterococci and Streptococci are usually tolerant for high NaCl concentration but lactococci mostly are moderately tolerant towards salt percentage. Lactococci have been shown to tolerate a salt concentration of 4.0 % but were found to be susceptible at 6.5% and 9.0 % concentration (Harris *et al*., 1992), although halophilicisolates of lactococci have been isolated from environmental samples, which survive a salt concentration of nearly 6 % (Itoi *et al*. 2008) [7].

Carbohydrate Utilization Test (Sugar Profiling)

Tentative identification of isolates was done mainly on the basis of carbohydrate utilization test (sugar profiling). The ability of the cultures to ferment and produce acid from various sugars was tested in Hi-Carbo Kit (Hi Media), which contained different sugars immobilized in wells. The cultures were grown in 10 ml M-17 broth at 30 ºC for 24 h and cells were harvested by centrifugation in refrigerated centrifuge. The supernatant was discarded carefully and pellet was washed using sterilized saline and re-suspended in 5 ml saline. The prepared inoculums were inoculated @ 50 µl in each well of the kit and incubated at 30 ºC for 24-48 h. The fermentation pattern obtained for different isolates and standard culture of Lactococci is given in Table 2. Out of 60 pure isolate s, all were confirmed Lactococci on the basis of polyphasic morphological and biochemical (catalase and sugar profiling test) identification. The color change of immobilized sugar in the well from red to yellow indicated the ability of the isolate to ferment the particular sugar. The glucose, fructose, ribose, trehalose, mannose, cellobiose and salicin. Maltose, galactose, raffinose, esculin, and arabinose were utilized by Lactococci strains, but sucrose, xylose, and lactose were not utilized by any of the isolates as energy sources. The organisms utilized L-malate and citrate in the presence of glucose, from which they produced D-(-)-lactate.

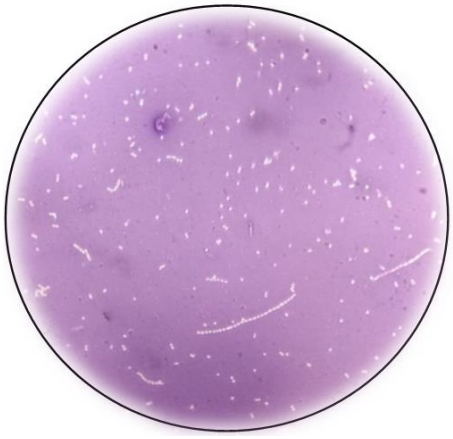

Fig 2: Negative Staining sample 9

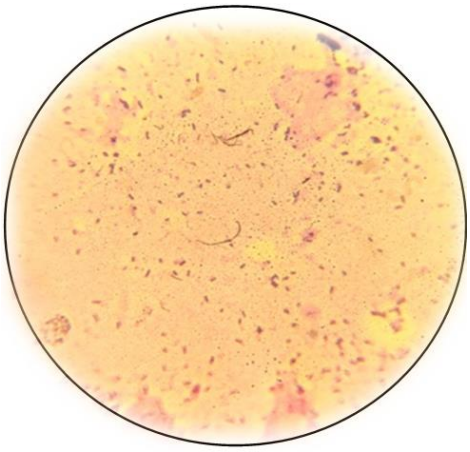

Fig 3: Gram staining sample 22

Table 2: Carbohydrate utilization of Lactococci

Isolates No.	Carbohydrates											
	Lac	**Xyl**	**Mal**	**Fru**	**Dex**	**Gal**	**Raf**	**Tre**	**Mel**	**Suc**	**L-ar**	**Man**
1,5,6,12,15,25,27,33,57	+	+	-	+	+	+/-	-	+	+	-	-	+
2,4,21,23,30,34,36,37,38,40,42,44,46,48,51,53,56	+	+	-	+	+	+	-	-	+	-	+	+
7,11,13,14,22,24,32,35,39,41,43,45,47,55,58	+	-	-	+	+	+	+	+	+	-	+	+
3,8,19,52,54	-	+	-	+	+	+	+	+	+	-	+	+
16,18,60	-	+	+	+	+	+	+	-	+	-	+	+
20,26,28,29,31	-	+	+	+	+	+	+	+	+	-	+	+
9,10,50,59	+	+	+	+	+	+	+	+	+	-	+	+

Mugula *et al*. (2003) [16] identified isolates from M-17 medium by utilizing the different sugars into acids using API kits. Saeed *et al*. (2013) [18] also stated that lactococci and streptococci were homolactic bacteria, because they ferment sugars mostly to lactic acid.

Fermentation of Oat Drink using Isolate Cultures

Altogether there are 60 isolates cultures that were inoculated with oat drink. To which 3% isolated culture was mixed and kept in incubator at 30 ˚C for 48 h. As a result, out of all the isolate cultures that were kept for ferment, 45 cultures were fermented with different acidity. During fermentation, the best ferment sample was no. 9, which was isolated from the *dahi* sample. Whose pH is 4.0 and acidity is 0.4

The physicochemical characteristics such as pH and titratable acidity (TA) are related to stability of bioactive components in plant derived products and not much difference observed with the values of all physicochemical parameters. Adding starter culture will cause decrease in pH and increase in titrable acidity at the end of the fermentation period due to acid productionn (Tomovska *et al*., 2016) [20]. The type of lactococci bacteria, fermentation time and their interaction significantly influences the pH (Gao *et al*., 2019) [3]. Studies illustrate (Shah, 2007) [19] that tolerable level of pH and titratable acidity for the fermented food products should range between 7–4.5 (pH) and 0.3–1.9% (TA)

Sensory Properties

The mean score for all the sensory characteristics such as colour, flavor, consistency, mouth feel, taste and overall acceptability was above seven- over nine-point scale for fermented oats drink. The mean total sensory score for fermented oat drink was higher. The study on sensory properties (Everitt, 2009) [2] reported that a mean liking score of 7 or higher on a nine-point scale is usually indicative of highly acceptable sensory quality, and a product achieving this score could be used confidently as a good illustration of "target" quality.

Conclusion

The isolated Lactococci strain used in this study possess good viability in oats drink and had good shelf life period of two weeks without addition of any preservatives. The developed product remained to be stable without phase separation throughout the course of storage period, had tolerable pH and titratable acidity, packed with essential proximate nutrients and other functional components such as b-glucan, antioxidant property and free fatty acids. It may also serve as a good substitute for serving individuals with lactose intolerance, gluten or casein sensitivities/allergic reaction and for vegans praising fermented oat substitutes. The observations depicted that microbial fermentation may be used as suitable and inexpensive technology in formulation of health promoting foods with functional components.

Acknowledgment

The author would like to express their thanks for the research, Vice-Chancellor of Dau Shri Vasudev Chandrakar Kamdhenu Vishwavidyalya, Raipur for financial support for this study.

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