An Official Journal of Bangladesh Livestock Research Institute (BLRI)
Bangladesh Journal of Livestock Research
Journal Homepage: https://www.banglajol.info/index.php/BJLR
ISSN 1022-3851

Isolation, identification and biochemical characterization of lactic acid bacteria from selected yogurt samples

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Abstract

This research was conducted to study the types of lactic acid bacteria (LAB) present in selected yogurts available in the local market of Bangladesh. For this purpose, nine different yogurt samples were collected (viz. MV, Mw, Pst, Psr, Bik, Bog, WF, Kw and Nab) and cultured in the selective MRS agar media for enumerating LAB colony. Out of 9 samples, colony forming LAB were found in 6 samples and the population ranged from 1.0×10^4 to 9.5×10^5 cfu/ml. Catalase negative and Gram's positive colonies were initially identified as LAB. Then the isolates were purified by subsequent culturing in MRS broth and MRS agar media. Biochemical properties of selected colonies were evaluated by performing gas production from glucose, growth at different temperatures (10ºC, 15ºC and 45ºC), growth at different NaCl concentrations (2, 4 and 6.5% NaCl) and sugar fermentation tests (lactose, sorbitol, salicin, trehalose, melibiose, sucrose, mannitol, melezitose, maltose, galactose, glucose, arabinose, raffinose and ribose). According to the tests stated above, a total of five different species of LAB were identified from 6 samples. The isolate *Lactobacillus lactis* was identified in MV, PSr and Bog yogurt, while *Lactobacillus bulgaricus* was found in MV and Bik yogurt. The species *Leuconostoc cremoris*, a avor producing bacteria, was found in six yogurt samples. On the other hand, *Lactobacillus acidophilus* and *Lactobacillus helveticus* was found only in Pst and Psr samples, respectively.

Key words: Yogurt, lactic acid bacteria, biochemical characterization.

Bang. J. Livs. Res. Vol. 27 (1&2), 2020: P. 64-72. https://doi.org/10.3329/bjlr.v27i1.55170

Introduction

Yogurt is a food obtained by control fermentation of milk by selective culture of lactic acid bacteria (LAB) being imparted

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flavor and typical aroma compound. It is easily digested and vital for health because of its high protein and fat content, lactose, minerals specially calcium and phosphorous, important vitamins and lactic acid (Ali *et al*., 2002). Yogurts may provide additional health benefits, for example it may reduce cholesterol levels and certain diseases with gastrointestinal tract such as lactose intolerance, diarrhea, colon cancer and other bacterial infection are inhibited through high consumption of yogurt (Hossain *et al*., 2015). Usually quality of yogurt depends on quality of starter culture. According to Food and Drug administration *Streptococcus thermophilus* and *Lactobacillus bulgaricus* is obligatory for yogurt starter culture but they also suggested some additional bacteria viz. *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus helveticus, Lactobacillus jugurti, Lactobacillus lactis, Bifidobacterium longum, Bifidobacterium bifidum and Bifidobacterium infantis* to use as starter culture bacteria (Hui, 1992).

In Bangladesh, traditionally yogurt is produced both in household and commercially for selling at market. The Starter culture used to produce these yogurt or types of microbes available in those marketed yogurt is not studied well. In some cases, microbial quality of yogurt in Bangladesh was not found satisfactory due to adulteration and poor hygienic condition of shops (Hassan *et al*.,2016). Moreover, shop keepers in urban areas usually preserve yogurt in freezer, which may hamper the viability of LAB in yogurt. As a result, the consumer may be depriving from obtaining desired LAB through yogurt. The present study would be able to provide information on the contents of LAB in some available yogurt samples. This may help to create consumer's awareness, using suitable starter bacteria and adoption of proper storage management from factory to retailer by the manufacturer and formulation of regulations for ensuring yogurt quality in terms of microbial contents.

Materials and Methods

Time and place of the Experiment

The present experiment was conducted at the Biotechnology Division of Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka-1341, from April 2017 to November 2018.

Sample Collection

Nine marketed yoghurt samples by different producers were collected from local market at Savar, Dhaka. These were MV, Mw, Pst, Psr, Bik, Bog, WF, Kw and Nab yogurt. Immediately after collection, samples were transferred at low temperature (using ice-box) to the laboratory. The pour-plate method was conducted immediately on the same day for LAB enumeration, colony morphology and isolation.

Enumeration of LAB

The enumerations of live colonies of LAB in the collected samples were performed by culturing bacteria on MRS agar media. Briefly, samples were homogenized and 3 ml of each sample was poured into a screw-cap tube containing 27 ml of 0.85% NaCl solution and vortexed to mix thoroughly. Considering this as the first dilution, ten-fold serial dilution was made. Hundred microliter (0.1 ml) aliquots from subsequent two dilutions (10^{-4} and 10^{-5}

dilutions) were poured on MRS agar media in two replicates. Inoculated plates were then incubated at 37°C for 48 hours in an incubator (Daihan, WIG-50, and Korea). After 48 h, visible colonies were counted from the plates at appropriate dilutions and the number of colony-forming units (cfu) was expressed per ml of sample.

Isolation and purification of LAB isolates

Isolation of bacteria was performed by pour plating technique using MRS agar media. This was done simultaneously during the step of enumeration. Initial identification was performed based on Gram's Staining and catalase test following the procedure described by Sharpe (1979). The colonies which were found white in color, Gram-positive and catalase-negative were considered as LAB (Sharpe, 1979). Then the LAB isolates were purified by culturing in MRS broth and MRS agar media sequentially until similar colonies were found. Finally, the purified isolates were identified by performing different biochemical tests.

Gram staining

Morphological status of isolated bacteria was determined using Gram staining. One ml of overnight activated broth culture was aliquoted into an Eppendorf tube and centrifuged at 6000 rpm for 5 min. The supernatant was removed and cells were re-suspended in sterile saline water. Ten microliters of cell suspension were pipetted to a microscopic slide and they were Gram-stained after drying and fixation by exposure to a flame. Briefly, staining was performed with crystal violet solution for 1 minutes as first step, followed by washing with tap water. Then iodine solution was placed on slide and kept for 1 minute followed by washing under tap water. Then 95% ethanol was used for 15 seconds until violet stain was removed from the slide. After removing ethanol by washing under tap water, slide was counterstained with safranine for 30 seconds . Finally, the slide was washed again under tap water and dried by using blot paper and observed under a microscope using 20x magnifications. Gram-positive organisms stained blue-purple, while, gram-negatives became pink-red after Gram staining (Erkuş, 2007).

Catalase test

Catalase enzyme breaks down hydrogen peroxide (H_2O_2) into oxygen and water molecules ($2H_2O_2$ $2H_2O + O_2$) and oxygen production is observed by the generation of O_2 bubbles. The generation of gas bubbles indicates presence of the enzyme, hence the catalase positive nature of the bacterium. A sterile loop was used to transfer a small amount of colony on the surface of a clean, dry glass slide. A drop of 3% H_2O_2 was placed right over the colony and observed for the evolution of oxygen bubbles (Reiner, 2010).

Gas production from glucose

Carbon di-oxide production from glucose is the major criterion for the determination of homofermentative or heterofermentative nature of an isolate. MRS broths containing inverted Durham tubes were utilized to determine CO2 production. Fifty micro litres of overnight activated cultures were inoculated into 8 ml MRS broth containing inverted Durham tubes in triplicates and incubated for 5 days at 37°C. The CO_2 production

was observed as an accumulation of gas in the inverted tubes.

Growth at different temperatures

To determine the growth at 10°C, 15°C and 45°C temperatures, the MRS broth media was prepared by adding bromecresol purple at a rate of 0.004%. Bromecresol purple was used to determine the color change from purple to yellow, indicating lactic acid production and cell growth. Fifty microliters of overnight activated culture were transferred into the tubescontaining 5 ml test media. After inoculation, they were incubated for 7 days at 10°C, 15°C and 45°C in duplicates. Cell growth at any of these temperatures was detected by the change in the color of the cultures, from purple to yellow.

Growth at different NaCl Concentrations

A 50µl of overnight activated culture was transferred into the tube containing 4.95 g of NaCl test media. The NaCl test media was prepared by adding 0.004% Bromcresol purple with required amount of NaCl in MRS broth media. Isolates were inoculated for testing growth at 2%, 4% and 6.5% NaCl solutions. They were incubated for 7 days at 37°C in the incubator. Changing color from purple to yellow has considered as evidence for cell growth.

Sugar fermentation test

The sugar (carbohydrate) fermentation tests were performed by inoculating bacterial culture into individual tubes containing sugars and incubated at 37ºC for 24 hours. Fourteen sugars e.g., glucose, lactose, sorbitol, salicin, trehalose, melibiose, sucrose, mannitol, melezitose, maltose, galactose, arabinose, raffinose and ribose were used for sugar fermentation test in this study. Firstly, 10% solution of each sugar was prepared in distilled water, with a gentle heat to facilitate dissolving completely followed by filtering through 0.2 microfilters. The stock solution was prepared by using 0.004% bromocresol purple with 0.85% NaCl and sterilized it by using autoclave at 121ºC for 15 minutes. Fifty microlitres of overnight activated culture were transferred into test tube containing 4ml stock solution with 950 µl specific sugar solution. At the same time sterilized Durham's tube was added inversely into the test tube to detect gas production. Acid production was indicated by the change of media color from purple to yellow while gas production was indicated by the appearance of gas bubbles in the inverted Durham's fermentation tubes.

Results and Discussion

Table 1 shows the results of LAB populations in different collected sample as expressed by colony-forming unit per millilitre of sample (cfu/ml). It was observed that MV yogurt sample showed higher LAB count (9.5×10^5 cfu/ml) and Mw yogurt sample showed lower number of LAB count (1.0×10^4 cfu/ml) among the samples. On the other hand, LAB was not observed in Nab, WF and Kw yogurt sample. Overall, lower concentrations of LAB was observed in all other collected samples. Hasan *et al*. (2016) observed standard plate count (SPC) in local Bogra yogurt sample was 3.47×10^7 cfu/ml while. Hossain *et al*., (2015) also reported that the range of SPC of different yogurt sample was 1.54×10^9 to 1.68×10^{12} cfu/ml.

However, in those studies, nutrient agar was used for counting bacteria, while in this study MRS agar was used, which allowed only LAB to grow on media. Live viable bacterial counts may differ sample to sample depending on duration and mode of preservation (e.g., refrigeration) by the retailers, other artificial treatments (e.g., UV treatment) by the manufacturer to make bacteria weakened or killed. Table 2 shows the results of catalase test, Gram staining and gas production test. As there were no live viable bacteria found in the sample of Nab, Kw and WF, they were not subjected for following tests regarding identification and characterization. A total of randomly selected 150 isolates from remaining 6 samples were considered for catalase test, Gram staining and gas production test. It was found that only 15 out of 150 isolates were lactic acid producing bacteria (LAB) according to the characteristics described by Sharpe (1979). Microscopic examination followed by Gram staining suggested that 7 isolates of LAB were rod shaped while the remaining 8 were cocci shaped bacteria. Further, gas production from glucose test revealed that all those rod shaped isolates were homofermentative, while cocci shaped isolates were heterofermentative. Based on these results, the isolates found catalase-negative, Gram positive, rod shaped and homofermentative identified as genus *Lactobacillus*. This was in with agreement with Abdullah *et al*. (2010), Erkus (2007), Erdourul and Erbulur (2006). On the other hand, isolates found catalase-negative, Gram-positive, cocci shaped and heterofermentative were identified under the genus *Leuconostoc*, which was in similar with Azadnia *et al*. (2009).

Table 1. Live viable count of LAB in collected yogurt samples

Name of Yogurt samples	LAB count(cfu/ml)
Bog	3.8×10^5
MV	9.5×10^5
PSt	3.8×10^5
PSr	4.0×10^4
Bik	5.0×10^4
Mw	1.0×10^4
Nab	n.d
Kw	n.d
WF	n.d

n.d =Not detected

The results of bacterial growth in different concentrations (2, 4 and 6.5%) of NaCl and at different temperatures (10, 15 and 45 ˚C) are presented in Table 3. Results showed that total 4 isolates (1 of MV and Bog and 2 of PSr yogurt) out of 15 have grown in all three concentrations of NaCl, and they also have grown at 45˚C, but not at 10 or 15˚C indicating their thermophilic nature. In contrary, total 8 isolates (1 from each MV, Mw , Bik and Bog and 2 from each PSt and PSr) did not grow in presence of NaCl in any of the concentrations tested, while these isolates grow at 10 and 15˚C, but not at 45˚C, indicating they are mesophilic.

Table 2. Characterization of isolates based on catalase test, Gram staining, microscopic examination and gas production from Glucose.

Yogurt name	MV			Mw	PSt			PSr				Bik		Bog	
Isolates	1	2	3	1	1	2	3	1	2	3	4	1	2	1	2
Catalase activity	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Grams stain reaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cell shape	Rod	Rod	Cocci	Cocci	Rod	Cocci	Cocci	Rod	Rod	Cocci	Cocci	Rod	Cocci	Rod	Cocci
Gas from Glucose	-	-	+	+	-	+	+	-	-	+	+	-	+	-	+
Fermentation type	Homo	Homo	Hetero	Hetero	Homo	Hetero	Hetero	Homo	Homo	Hetero	Hetero	Homo	Hetero	Homo	Hetero
Genus	*Lactobacillus*	*Lactobacillus*	*Leuconostoc*	*Leuconostoc*	*Lactobacillus*	*Leuconostoc*	*Leuconostoc*	*Lactobacillus*	*Lactobacillus*	*Leuconostoc*	*Leuconostoc*	*Lactobacillus*	*Leuconostoc*	*Lactobacillus*	*Leuconostoc*

Table 3. Growth of isolates found in different yogurt samples at different NaCl concentration and temperatures

Yogurt name	MV			Mw	PSt			PSr				Bik		Bog	
Isolates	1	2	3	1	1	2	3	1	2	3	4	1	2	1	2
Growth in different NaCl solution															
2% NaCI	+	+	-	-	+	-	-	+	+	-	-	+	-	+	-
4% NaCI	+	-	-	-	-	-	-	+	+	-	-	-	-	+	-
6.5% NaCI	+	-	-	-	-	-	-	+	+	-	-	-	-	+	-
Growth at different temperature															
at 10˚ C	-	-	+	+	-	+	+	-	-	+	+	-	+	-	+
at 15˚ C	-	-	+	+	-	+	+	-	-	+	+	-	+	-	+
at 45˚ C	+	+	-	-	+	-	-	+	+	-	-	+	-	+	-

On the other hand, 3 remaining isolates (1 from each MV, PSt and Bik) out of 15 were also found thermophilic in nature as they did grow at 45˚C and only in 2%, but not in 4 and 6.5% NaCl solution. The present results regarding heat tolerance characteristics of isolates can be explained with many other previous findings (Ammor *et a*l., 2005; Guessas *et al*., 2004; Hemme *et al*.,2004; Busson *et al*., 1999; Samelis *et al*., 1994).

Table 4 shows the sugar fermentation characteristics of isolates found in different yogurt samples. In this study, sugar fermentation tests included 14 sugars, which were, lactose, sorbitol, salicin, trehalose, melibiose, sucrose,

mannitol, melezitose, maltose, galactose, glucose, arabinose, raffinose and ribose. Total 8 isolates (1 from each MV, Mw, Bik and Bog, 2 from each PSt and PSr) were observed to ferment only glucose and galactose among 14 sugar tested. These findings, in combinations with the results from Table 2 and 3 indicated that the isolate was *Leuconostoc cremorisas* also stated by Azadnia and Khan Nazer (2009).The *Leuconostocs* are Gram-positive cocci, occurring in pairs and chains, and form D (–) lactic acid and carbon dioxide from the fermentation of glucose indicating heterofermentative-type metabolism (Azadnia and Khan Nazer, 2009). The *L. cremoris* is biochemically differentiated by its inability to ferment trehalose and sucrose but it ferments glucose, galactose, and lactose (Azadnia and Khan Nazer, 2009).

A total of 3 isolates (1 from each MV, PSr and Bog) were found to able ferment lactose, salicin, trehalose, maltose and glucose. These characteristics, in combination with other biochemical properties as presented in Table 2 and Table 3 indicated that the isolate is *Lcatobacillus lactisin* accordance with (Abdullah *et al*., 2010) and Erkus (2007). The *L. Lactis* is catalase negative, Gram positive, rod shaped, thermophilic bacteria (grows at 45^{0}C), which form gas from glucose and can grow in 2, 4 and 6.5% NaCl solution (Erkus, 2007). These bacteria ferment lactose, salicin, trehalose, maltose and glucose but unable to ferment sorbitol, melibiose, melibiose, sucrose, mannitol, galactose, arabinose, ribose and raffinose (Erkus, 2007).

Two other isolates (1 from each MV and Bik) did ferment only glucose and lactose among the 14 sugars tested and the characteristics are inconsistent with *Lactobacillus bulgaricus* as described by (Abdullah *et al*., 2010) and Erkus
(2007). This species was described as catalase negative, Gram positive, rod shaped and had the ability to form gas from glucose. It is a thermophilic bacteria (grows at 45^{0}C), which can grow in 2% NaCl but not in 4 and 6.5% NaCl. The *L. bulgaricus* ferments lactose and glucose but not salicin, trehalose, sucrose, maltose, sorbitol, melizitose, mellibiose, arabinose, ribose, raffinose, galactose and mannitol (Mithun *et al*, 2015; Erkus, 2007).

One isolate from PSt sample was identified as *Lactobacillus acidophilus* as it was found to ferment lactose, salicin, trehalose, sucrose, maltose and glucose but were unable to ferment sorbitol, melibiose, mannitol, melezitose, arabinose, raffinose and ribose. These sugar fermentation characteristics of *L. acidophilus* are in agreement with Mithun *et al*., 2015), Abdullah *et al*., 2010 and Erkus (2007). The *L. acidophilus* was characterized as catalase negative, Gram positive, rod shaped and had the ability to form gas from glucose (Heterofermentative), thermophilic in nature (grows at 45^{0}C), which can grow in 2% NaCl but not in 4 and 6.5% NaCl (Mithun *et al*, 2015; Erkus, 2007).

One isolate from PSr was found to ferment lactose, sorbitol, salicin, trehalose, sucrose, mannitol, melizitose, maltose, glucose and galactose, but not the mellibiose, arabinose, raffinose and ribose and can be identified as *Lctobacillus helveticus* according to Erkus (2007). The *L. helveticus* is catalase negative, Gram positive, rod shaped and produce gas from glucose (heterofermentative). It grows in 2, 4 and 6.5% NaCl solution. It also grows at 45^{0}C but not at 10^{0}C and 15^{0}C (Mithun *et al*, 2015; Erkus, 2007).

Table 4. Carbohydrate fermentation characteristics of isolates obtained from different yogurt samples

Sample ID	MV			Mw	PSt			PSr				Bik		Bog	
Isolates	1	2	3	1	1	2	3	1	2	3	4	1	2	1	2
Lactose	+	+	-	-	+	-	-	+	+	-	-	-	-	+	-
Sorbitol	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Salicin	+	-	-	-	+	-	-	+	+	-	-	-	-	+	-
Trehalose	+	-	-	-	+	-	-	+	+	-	-	-	-	+	-
Mellibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-
Manitol	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Maltose	+	-	-	-	+	-	-	+	+	-	-	-	-	+	-
Galactose	-	-	+	+	-	+	+	-	+	+	+	-	+	-	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ribose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Species	*Lactobacillus lactis*	*Lactobacillus bulgaricus*	*Leuconostoc emoris*	*Leuconostoc emoris*	*Lactobacillus acidophilus*	*Leuconostoc emoris*	*Leuconostoc emoris*	*Lactobacillus lactis*	*Lactobacillus helveticus*	*Leuconostoc emoris*	*Leuconostoc emoris*	*Lactobacillus bulgaricus*	*Leuconostoc emoris*	*Lactobacillus lactis*	*Leuconostoc emoris*

Conclusion

In can be concluded that total of five different species of LAB were identified from six local yogurt samples. The isolate *Lactobacillus lactis* was identified in MV, PSr and Bog yogurt, while *Lactobacillus bulgaricus* was found in MV and Bik sample. The species *Leuconostoc cremoris*, a flavor producing bacteria in yogurt, was found in all six yogurt samples containing live viable colonies. On the other hand *Lactobacillus acidophilus* and *Lactobacillus helveticus* was found only in PSt and PSr yogurt samples.

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