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# Isolation and Characterization of Lactic acid Bacteria from Indigenous Dairy Product and Preparation of Starter Culture by Freeze-drying

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**ABSTRACT:** The quality of curd varies widely within different regions of Bangladesh which primarily depends on the starter cultures that are categorized in the group of lactic acid bacteria (LAB). Maintenance, preservation and use of quality starter cultures are prerequisite for quality curd production which is poorly maintained in the country. To explore the potential and quality starter culture and preserve these starter culture with freez-drying method, different curd samples from local popular brands were collected, processed and inoculated on MRS agar. For isolation and characterization of LAB isolates, a total of 12 distinct isolates were picked up from MRS agar, subcultured to pure culture and subjected to different biochemical, cultural and morphological examinations. Among the 12 isolates, 5 different species of *Lactobacillus* were identified as *L. acidophilus, L. casei, L. brevis, L. viridescens,* and *L. bulgaricus.* Among these, *L. brevis* and *L. acidophilus* were freeze-dried using four different cryoprotectiveagents viz. glycerin, mannitol, sorbitol, and skim milk. Viability of these freeze dried isolates was determined immediately after the freeze drying and after one month of storage at 4°C. In all cases there was approximately one log decrease in the cell count after freeze-drying. Viability of *L. acidophilus* after one month was decreased approximately by two logs, although viability for the *L. brevis* decreased to a greater extent. The highest viability was found in *L. brevis* that was freeze-dried in glycerin and the lowest viability was found in *L. acidophilus* that was freeze-dried in mannitol. Starter potential of those freeze-dried starter culture was confirmed by curd formation with those isolates using skim milk and liquid milk as substrate. It can be concluded from our study that the isolated LAB can be used as potential starter culture for curd production and can be preserved successfully by freeze-drying for more than one month stored at 4°C.

**KEYWORDS:** Curd, *Lactobacillus*, Freeze-dry, Starter culture, Culture viability

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## INTRODUCTION

In Bangladesh curd (Dadhi) is one of the most popular fermented dairy product made by milk with lactic acid bacteria (LAB)[1], which is consumed throughout the country. Curd provides numerous beneficial effect to human and other animal health such as providing a beneficial microflora in the intestine[2], enhancing clinical recovery from diarrhea of children caused by rotavirus[3], decreasing serum concentration of VLDL and IDL[4]. It has been also reported that calcium and phosphorus content of

curd can be more easily absorbed compared to milk[5].

Curds are prepared in different areas of Bangladesh usually by backslopping i.e., previously prepared curd as starter culture. As different types of cultures are used in different parts of Bangladesh, the quality of the curds varies enormously[6]. In previous study it has been reported that the quality of curd which are available in local market of Bangladesh are variable and the curd produced in Bogura is observed as the best quality of curd[7].

Sometimes curd formation is completely prevented by bacteriophages that are present in the milk. Bacterial strains that are highly sensitive to bacteriophages can completely abrogate the formation of curd. A potential bacterial strain with resistance to bacteriophages, and longer viability, can solve the previously described problems in curd industry[8].

So far from our knowledge, no standard starter culture is maintained or preserved to make quality curd in Bangladesh. Therefore, to improve the curd quality is the main focus of our present study by isolating and identifying potential LAB and preserving them using suitable method. Some LAB can harbor mobile antibiotic resistance genes and these genes can be horizontally transferred to commensal and pathogenic microorganism of the intestine which assumes to be very threating for the treatment of disease[9]. The traditional starter culture may become contaminated with pathogenic and antibiotic resistant bacteria and these pathogenic bacteria can be transferred to the subsequent curd production. This is why, preserving pure starter culture with suitable method is very important in quality curd production. Bacterial culture can be preserved by various ways e.g., conventional liquid-state, solid state or power form by freeze drying etc. In conventional liquid-state culture technique, viability of LAB can be lost easily within 1-2 weeks even after maintaining at 4-7°C[10]. On the contrary, freeze-drying method is one of the easiest, economic and convenient ways[11] to preserve different culture for long time. For this reason, we isolated some LAB from different curd samples and prepared freeze-dried starter culture with our isolated selected strains using some cryoprotective agents. After freeze drying, viability of these freeze dried culture was assessed and found to maintain the viability at satisfactory level after one month at 4°C.

## METERIALS AND METHODS

### Collection of Samples:

Curd samples were collected from six local brands named Bogura, Muslim, Bonoful, Savar, Jagadish and Sadapur, and designed as Bg, Mu, Bo, Sa, Ja and Sd, respectively. Samples were collected in sterile beaker from different retail shops of Dhaka, Savar and Bogura and brought to Microbiology and Industrial Irradiation Division (MIID) laboratory, AERE, Savar, Dhaka as soon as possible. After collection, samples were stored immediately in aseptic condition of 4˚C temperature to protect from contamination and deterioration.

### Isolation of Lactobacillus species:

*Lactobacillus* spp. were isolated from curd samples by using MRS (Man, Rogosa, and Sharpe) agar medium[12], supplemented with 0.5% $CaCO_3$ to improve the specificity of the medium. The pHof the media was adjusted to 6.5.

Ten grams of each sample was suspended in 90 ml normal saline, which was then shaken homogenously, and serially diluted and plated on MRS agar plates. The plates were incubated at 37°C for 24 h in anaerobic condition. After incubation, the bacterial colonies were restreacked on the MRS agar with 0.5% $CaCO_3$ media for the formation of clear zone. The bacterial load of each curd samples were observed by counting the serial plate ($10^{-1}$ to $10^{-6}$ dilution).

### Identification of LAB:

Characterization of all the isolates was performed on the basis of their morphological and biochemical characteristics. Morphological examinations were carried out by using Gram's staining method[13]. Pure culture on MRS agar plate was identified by biochemical test like catalase test, sugar fermentation test, NaCl tolerance and temperature tolerance test[14].

For NaCl tolerance test, fresh overnight cultures of *Lactobacillus* isolates were transferred to tubes containing MRS broth with different concentrations of NaCl. After sterilization, and incubated at 37°C for 24 h. Maximum growth (turbidity) was indicated as double positive sign (++), normal growth were indicated as single positive sign (+), and no growth were indicated as negative sign (-).

For the determination of temperature tolerance *Lactobacillus* isolates were incubated at 10°C, 40°C and 45°C for 24 h. After 24 hours of incubation

their growth were determined by observing their turbidity.

**Preservation of LAB by Freeze-Drying:**
Pure cultures of LAB were freeze-dried by using Freeze-Dryer machine. Cells were harvested by centrifugation at 5000×g for 10 min at 20°C and weighted in four 100-ml freeze-drying bottle. The cell mass mixed with 4 different protective agents (glycerin, mannitol, sorbitol, and skim milk) in a ratio of 1:5. Sterile suspension of protectants was prepared in phosphate buffered saline (PBS) solution of the concentration at 10% (w/v). Samples were dried in a freeze-Dryer with a condenser temperature of -52°C and a chamber pressure $p \leq 0.08$ mbar for 48 h[15].

**Viability during Storage:**
Freeze-dried cultures were stored in sterile polyethylene packets and placed at 4°C to evaluate the stability. The viability of the freeze-dried samples was performed using spread plate count techniques at production day and one month storage and freeze-dried *Lactobacillus* culture was used to prepare the curd.

**Determination of Lactic Acid Production:**
Ten grams curd was mixed with 30 ml distilled water and acidity was determined by titration with 0.1 *N*NaOH. pH of the separated liquid was recorded using a digital electrode pHmeter.

## RESULTS AND DISCUSSION

**Morphology and cultural phenotypes of isolates:**
From the tested samples, twelve presumptive lactic acid producing isolates were picked up. Microscopic observation revealed that all the isolates were rod shaped, and all showed positive results when tested for Gram reaction **(Table S1)**. From this results we could assume that these isolates could be the members of *Lactobacillus*[16].
The cultural characteristics of the isolates were determined on the basis of their colony colour, colony size, colony shape and bacterial consistency on the MRS agar plates. Colony colours differed from sample to sample and most predominant colors found were whitish to brownish although some yellowish colonies were found in sample collected from Bg. Colony size were also variables from sample to samples and most of the colonies are in small to medium in size. Colony shapes from all samples were circular and consistency of the colonies of all bacterium was sticky in nature **(Table S1)**. All the isolates form clear zone formation around the colonies on MRS medium containing $CaCO_3$, which is very similar to another study where oral and fecal lactobacillus strains were isolated[12].

**Growth at different temperature and various NaCl concentrations:**
None of the isolates could grow at 10°C or could tolerate 10% of NaCl concentration. All isolates could grow easily at 4-6% of NaCl **(Table S2)**, which is in agreement with the other studies[17]. The maximum growth rate of Mu2 and Mu4 isolates were observed in 45°C, while the other isolates had an optimum temperature around 40°C.

**Biochemical characteristics for the isolates:**
All isolates revealed negative results when they were tested for catalase enzyme test and none of the isolates produced gas from glucose during fermentation process in Durham's tube. In a different study from the goat's milk in Algerian arid zone showed the same result for the catalase test and gas formation from glucose[18]. Among the bacterial isolates, one was identified as *L. brevis*, two were identified as *L. bulgaricus*, three were dentified as *L. viridescens*and another three were as *L. casei*, and three were identified as *L. acidophilus* **(Table S3)**. It was also observed that samples from Bogura, Muslim and Bonoful contained mixed culture of LAB, while for other samples we could only isolate single *Lactobacillus* strain.

**Effect of freeze-drying on viability of Lactobacillus:**
In order to preserve the selected isolates (i.e. *L. brevis*, *L. acidophilus)* for a longer time, the effect of the freeze-drying on the viability of each selected strains with different protectants was studied. The results of this experiment were summarized in **Table S4**. *L. brevis* showed highest viability in glycerin ($1.24\times10^{10}$cfu/g to $1.0\times10^{9}$cfu/g) and *L. acidophilus* in skim milk ($2.165\times10^{9}$cfu/g to $2.88\times10^{8}$cfu/g). *L. acidophilus* showed poor or no viability in case of mannitol. Therefore, glycerin and skim milk can be used as protectants when *Lactobacillus* isolates are freeze-dried as a starter culture to produce curd.

**LAB viability after a storage period of one month:**
There was a slight decrease in the bacterial count after one month of freeze-drying in the case of *L. acidophilus* as compared to *L. brevis*. *L. brevis*

decreased to 4 to 5 log in all the protectant after freeze-drying. *L. acidophilus* was nonviable in case of glycerin. The inability to strictly maintain the temperature for the freeze-dried sample might be a cause for this no viability. Viability of freeze dried *Lactobacillus* after one month was variable and it also relied on protectants that were used during freeze-drying process. Skim milk was best protectant for the preservation of both species of *Lactobacillus.*

**Assessment of curd formation:**

The curd formation capabilities of dried starter culture were observed both in skim milk and cow milk. The organoleptic characters of prepared curd samples were summarized in **Table S5.** All starter cultures were able to form curd in skim milk except for *L. brevis*, which could not produce curd in liquid milk. All prepared curds were organoleptically accepted by a panel with respect to their texture, color, and flavor.

**Assessment of Lactic Acid Production:**

In this study *L. acidophilus* produced acid in both curds from skim milk and liquid milk and the measured titratable acidity was 0.57% in skim milk 0.501% in liquid milk. *L. brevis* could not make curd from liquid milk. In skim milk *L. brevis* was able to form curd and the titratable acidity was found as 0.46% **(Table S6)**.

Based on the finding of the present study, it is concluded that isolated LAB are potential for curd production and these LAB prepared by freeze drying can be preserved at least one month and from this preserved starter culture curd can be successfully made in Bangladesh.

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**Table S1: Morphological and cultural characterization of isolated bacterial strains**

| Sample code | No. of isolates | Morphological characteristics | | Cultural characteristics | | | |
|---|---|---|---|---|---|---|---|
| | | Gram staining | Shape | Colony color | Colony size | Colony shape | Consistency |
| Bg | Bg1 | Gram (+)ve | Rod | Yellowish | Pinpoint | Circular | Sticky |
| | Bg3 | Gram (+)ve | Rod | Whitish | Small | Circular | Sticky |
| Mu | Mu2 | Gram (+)ve | Rod | Whitish | Small | Circular | Sticky |
| | Mu4 | Gram (+)ve | Rod | Creamy | Small | Circular | Sticky |
| Bo | Bo2 | Gram (+)ve | Rod | Whitish | Small | Circular | Sticky |
| | Bo5 | Gram (+)ve | Rod | Brownish | Pinpoint | Circular | Sticky |
| Sa | Sa1 | Gram (+)ve | Rod | Brownish | Small | Circular | Sticky |
| | Sa2 | Gram (+)ve | Rod | Brownish | Small | Circular | Sticky |
| Ja | Ja1 | Gram (+)ve | Rod | Brownish | Small | Circular | Sticky |
| | Ja2 | Gram (+)ve | Rod | Whitish | Small | Circular | Sticky |
| Sd | Sd1 | Gram (+)ve | Rod | Whitish | Medium | Circular | Sticky |
| | Sd2 | Gram (+)ve | Rod | Whitish | Small | Circular | Sticky |

**Table S2: Growth of isolates at different temperature and NaCl concentration**

| Sample code | No. of isolates | Temperature Tolerance Test | | | NaCl Tolerance Test | | | |
|---|---|---|---|---|---|---|---|---|
| | | 10°C | 40°C | 45°C | 4% | 6% | 8% | 10% |
| Bg | Bg1 | - | ++ | + | ++ | ++ | + | — |
| | Bg3 | - | ++ | + | ++ | ++ | + | — |
| Mu | Mu2 | - | ++ | ++ | ++ | ++ | + | — |
| | Mu4 | - | ++ | ++ | ++ | ++ | + | — |
| Bo | Bo2 | - | ++ | + | ++ | ++ | + | — |
| | Bo5 | - | ++ | + | ++ | ++ | + | — |
| Sa | Sa1 | - | ++ | + | ++ | ++ | + | — |
| | Sa2 | - | ++ | + | ++ | ++ | + | — |
| Ja | Ja1 | - | ++ | + | ++ | ++ | + | — |
| | Ja2 | - | ++ | + | ++ | ++ | + | — |
| Sd | Sd1 | - | ++ | + | ++ | ++ | + | — |
| | Sd2 | - | ++ | + | ++ | ++ | + | — |

**Table S3: Biochemical test results of isolated bacterial strains**

| Sample code | No of isolates | Catalase test | Gas from glucose | Fermentation of sugar | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Lactose | galactose | Sucrose | Maltose | Mannitol | Rhamnose | Arabinose | Cellobiose | Sorbitol | Species |
| Bg | Bg 1 | - | A | + | + | + | + | + | - | + | + | - | *L. acidophilus* |
| | Bg 3 | - | A | + | + | + | + | + | - | - | + | + | *L. casei* |
| Mu | Mu2 | - | A | - | - | - | + | - | - | + | - | - | *L. brevis* |
| | Mu4 | - | A | + | + | + | + | + | - | + | + | - | *L. acidophilus* |
| Bo | Bo2 | - | A | + | + | + | + | + | - | + | + | - | *L. acidophilus* |
| | Bo5 | - | A | - | - | - | + | + | - | - | - | - | *L. viridescens* |
| Sa | Sa1 | - | A | + | + | - | + | - | - | - | - | - | *L. bulgaricus* |
| | Sa2 | - | A | + | + | - | + | - | - | - | - | - | *L. bulgaricus* |
| Ja | Ja1 | - | A | + | + | + | + | + | - | - | + | + | *L. casei* |
| | Ja2 | - | A | + | + | + | + | + | - | - | + | + | *L. casei* |
| Sd | Sd1 | - | A | - | - | - | + | + | - | - | - | - | *L. viridescens* |
| | Sd2 | - | A | - | - | - | + | + | - | - | - | - | *L. viridescens* |

**Table S4: Effect of freeze-drying on the viability of selected strains**

| Strains | Protective agents | B.C before F.D (cfu/g) | B.C after F.D (cfu/g) | B.C After one month of F.D(cfu/g) |
|---|---|---|---|---|
| *L. brevis* | Glycerin | $1.24\times10^{10}$ | $1.0\times10^{9}$ | $2.9\times10^{5}$ |
| | Mannitol | | $4.02\times10^{8}$ | $8.7\times10^{3}$ |
| | Sorbitol | | $3.2\times10^{8}$ | $3.8\times10^{4}$ |
| | Skim milk | | $2.17\times10^{8}$ | $1.8\times10^{4}$ |
| *L. acidophilus* | Glycerin | $2.165\times10^{9}$ | $1.19\times10^{6}$ | No growth |
| | Mannitol | | No growth | No growth |
| | Sorbitol | | $1.04\times10^{8}$ | $2.1\times10^{7}$ |
| | Skim milk | | $2.88\times10^{8}$ | $3.2\times10^{6}$ |

**Table S5: Organoleptic characters of the curd samples prepared by Freeze-dried culture**

| Strains | Types of Milk | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Skim milk | | | | Liquid milk | | | |
| | Color | Texture | Taste | Flavor | Color | Texture | Taste | Flavor |
| *L. brevis* | White | Semi-solid | Sour | Natural curd flavor | - | - | - | - |
| *L. acidophilus* | White | Thick | Sour | Natural curd flavor | White | Semi-solid | Sour | Natural curd flavor |

**Table S6: Lactic acid production in prepared curd samples**

| Strains | pH | | Titratable acidity (%) | |
|---|---|---|---|---|
| | Skim milk | Liquid milk | Skim milk | Liquid milk |
| *L. brevis* | 4.3 | 6.68 | 0.46 | - |
| *L. acidophilus* | 3.97 | 4.10 | 0.57 | 0.501 |