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STUDIES ON YOGURT PRODUCTION USING *Lactobacillus bulgaricus* AND *Streptococcus thermophilus* ISOLATED FROM MARKET YOGURT

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Abstract

Yogurt is a fermented milk product obtained from fermentation of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* strains. The aim of this study was to produce yogurt using isolated native starters *Lactobacillus delbruckii* sub sp. *Bulgaricus* and *Streptococcus saliverius* sub sp. *thermophilus.* In this study yogurt samples were collected from the local markets. The native starters were isolated, purified and identified according to Bergey's manual of determinative bacteriology. *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were screened with respect to their acid and flavour production for the preparation of a natural yogurt and compared to a commercial starter cultures. Yogurt was made by inoculating isolated bacteria (starter culture) usually *Lactobacillus delbruckii* subsp. *bulgaricus* and *Streptococcus saliverius* subsp. *thermophilus* into milk. After inoculation the mixture was incubated at 42°C for 6 to 8 hours and coagulation was observed and yogurt pH was measured. It is well known that yogurt production using native starter cultures instead of commercial ones is beneficial in respect of both economic and organoleptic aspects. The present results indicated that the isolated native yogurt starters can be used in yogurt manufacturing industry at a large scale.

***Keywords*:** Yogurt, starter culture, fermented product, *Lactobacillus bulgaricus*, *Streptococcus thermophilus.*

Introduction

Yogurt locally known as dahi in Bangladesh is a very popular fermented dairy product obtained from the milk or the milk products by the lactic acid fermentation through the action of *Streptococcus salivarius* sub sp. *thermophilus*, *Lactobacillus delbrueckii* sub sp. *bulgaricus* (Yilmaz-Ersan and Kurdal, 2014). Yogurts vary in appearance, flavor and ingredients. Various combinations of starter cultures were selected during manufacturing of yogurt to achieve desirable characteristics of product and also to provide the consumers with a wide choice of therapeutic benefits. Depending on its activity, manufacturer usually adds 2 to 4% yoghurt starter culture (Aswal*et al.*, 2012). There is a symbiotic relationship between the two species of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* that's why there is more rapid acid development than in the single strain culture (Rasic and Kurmann, 1978; Tamime and Deeth, 1980). In Bangladesh, yogurt is perhaps the oldest fermented milk product known and consumed by large parts of the population as a part of their daily diet. In most of the areas of Bangladesh, different types of traditional yogurts are found. Dahi or in other word yogurt are prepared almost country wide. But specific regional made yogurt (from Bogra district) are famous for their taste, texture, color and public preference. They uses various types of ingredients other than starter culture which directly affect the taste and other quality parameters. But the main role played for yogurt production is the different types of bacteria which directly affect their quality. Yogurt contains thermophilic starter culture such as *Lactobacillus delbruckii* sub sp. *bulgaricus* and *Streptococcus saliverius* sub sp. *thermophilus. Lactobacillus delbruckii* are rod with rounded ends shape but *Streptococcus thermophilus* has a spherical to ovoid shape with an irregular segments. Both are gram positive, facultative anaerobic, non-motile and non-sporeforming bacteria. Successful preparation of yogurt depends upon the proper symbiotic relationship between the two organisms at equal proportion. Therefore, the present study was undertaken for isolation and identification and antibiotic susceptibility test of *Lactobacillus delbruckii* sub sp. *Bulgaricus* and *Streptococcus*

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saliverius sub sp. *thermophilus* and finally, production of yogurt from isolated native starters *Lactobacillus delbruckii* sub sp. *bulgaricus* and *Streptococcus saliverius* sub sp. *thermophilus.*

Materials and Methods

Sample collection

The samples of fresh yogurt (curd) were collected from commercial market of Sylhet district and aseptically transferred to the Laboratory of the Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology for studies. The samples were stored under refrigeration conditions for subsequent experiments.

Sample preparation

Lactic acid bacteria (LAB) isolates were selected from (De Man Rogosa Sharpe) MRS and M17-agar plates of the highest dilutions. The isolates were sub cultured in MRS and M17-broth and streaked onto MRS-agar until pure cultures were obtained (Fadela *et al.*, 2008). Diluted samples (0.1 ml) were plated (spread plate technique) onto MRS medium for *Lactobacillus* isolation and M17 medium for *Streptococcus* isolaton and incubated at 37ºC for 24 to 72 hrs.

Identification and characterization of bacteria

Morphological characterization: Morphological appearance of colonies of lactic acid bacteria were observed carefully. Morphological characteristics that were studied after 24 hours were shape, size, colour, texture, surface, opacity, edge etc.

Biochemical characterization**:** Biochemical studies were performed according to the Bergey, 2006. Several biochemical tests were performed for confirmation of *Lactobacillus bulgaricus* and *Streptococcus thermophilus.* The biochemical tests were sugar fermentation tests, indole test, Methyl Red (MR) test, Voge's Proskauer (VP) test, Oxidase test, Catalase test, Oxidative-Fermentative test (O-F test). These species were characterized by their ability to ferment glucose, maltose, lactose and color changed but not gas formed, negative for indole test, positive for MR test and negative for VP, Oxidase test and catalase test, fermentative for O-F test.

Assay of antibiotic sensitivity pattern: To assay the antibiotic sensitivity pattern disc diffusion method was followed. In this method Muller Hinton plates were prepared and swabbed with suspension of selected isolates with the help of sterile cotton bud. MRS agar was used since the *Lactobacillus sp*. failed to thrive on Muller Hinton Agar which is the conventional medium for test (Okafor and Umeh, 2013). After swabbing the antibiotic discs (ampicillin, tertracycline, gentamycine, azithromycin, chrolamphenicol and ciprofloxacin) were placed on the surface of the plates at equidistance. The plates were then kept at 4°C for 1 to 2 hours for proper disc diffusion of antibiotics. The plates were then incubated for 18 to 24 hours at 37°C. The zone of inhibition was observed for antibiotic sensitivity or resistance and zone diameter was measured (Bauer *et al*., 1966).

Results and Discussion

Bacteria isolated from yogurt were identified as *Lactobacillus delbruckii* sub sp. *bulgaricus* and *Streptococcus saliverius* subsp. *thermophilus* by observing their colony morphology, physiological and as well as some biochemical characteristics. To analysis all bacterial isolates, it was confirmed that 11 isolates were *Streptococcus saliverius* subsp. *thermophilus* and 6 isolates were *Lactobacillus delbruckii* sub sp. *bulgaricus* among finally selected 17 isolates. List of presumptive bacteria isolates (Table 1) were identified according to Bergey (2006). Microscopically they were gram-positive, rod shaped (Fig. 1), coccus shape (Fig. 2), non-motile, Catalase negative, Oxidase negative, Indole negative, MR positive, VP negative, fermentative for OF test. They had the ability to ferment glucose, maltose, lactose.

The pattern of resistance or sensitivities of a given microorganism towards a range of antibiotics is defined as antibiogram. An antibiotic is a substance that is produced by a microorganism and at low concentrations inhibits or kills other microorganisms (Guardabassi and Courvalin, 2006 and Levy, 1998). In the present study, antibiotic susceptibility test was performed for identified 6 *Lactobacillus delbruckii* sub sp. *bulgaricus* isolates and 11 *Streptococcus saliverius* sub sp. *thermophilus* isolates by using 5 different antibiotic discs such as Ampicillin (AM), Gentamycine (GM), Ciprofloxacin (CIP), Tetracycline (TE), Azithromycin (AZ). Table 2 showed the sensitivity

pattern (at different concentrations) against antibiotics. In present study, all the 17 isolates show resistance to AM (100%), CIP (94.12%), TE (100%), AZ (100%), GM (82.35%). In the present study (Table 3), all the *Streptococcus saliverius* sub sp. *thermophilus* isolates showed 100% resistance to Ampicillin (AM), Gentamycine (GM), Ciprofloxacin (CIP), Tetracycline (TE), Azithromycin (AZ). In present study (Table 4 and Fig. 3), all the *Lactobacillus delbruckii* sub sp. *bulgaricus* isolates showed resistance to GM (50%), AM (100%), TE (100%), CIP (83.33%), AZ (100%) and they showed intermediates against GM (16.33%) and CIP (16.33%) and they also showed 33.33% susceptibilities against GM.

Table 1. List of presumptive isolates

Sample code	Name of bacteria	Number of isolates
LB-1, LB-2, LB-3, LB-6, LB-10, LB-11, LB-12, LB-13,LB-14, LB-15, LB-16	*Streptococcus saliverius* subsp. *thermophiles*	11
LB-4, LB-7, LB-8, T-6, T-7, T-8	*Lactobacillus delbruckii* subsp. *bulgaricus*	6

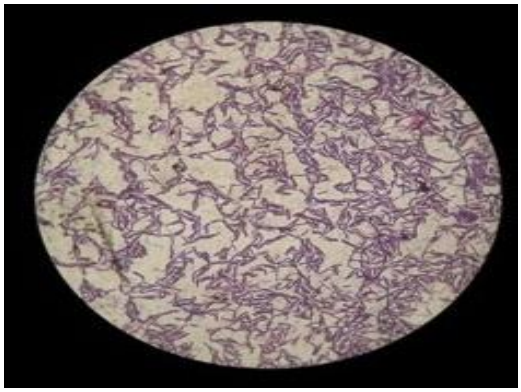

Fig. 1. Isolated rod shaped *Lactobacillus delbruckii* subsp. *bulgaricus*

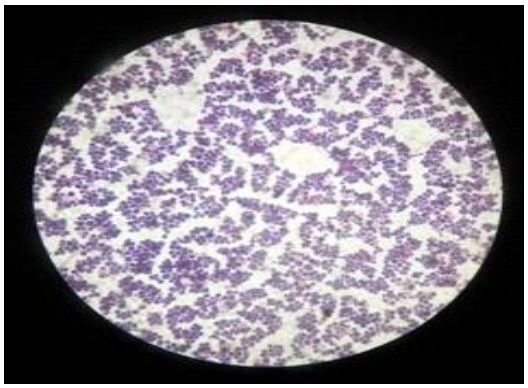

Fig. 2. Isolated spherical to ovoid shaped *Streptococcus saliverius subsp. thermophiles*

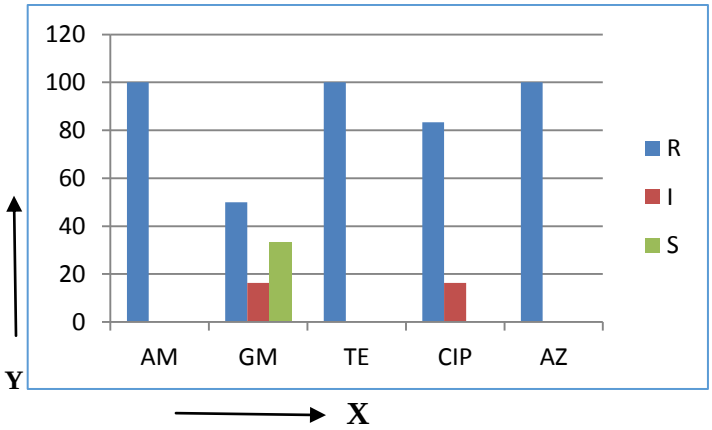

Fig. 3. Percentage of antibiotic Resistance (R), Intermediate (I) and Sensitivity (S) among the 6 isolates of *Lactobacillus bulgaricus*

Table 2. Antibiotic sensitivity pattern of the selected isolates

Isolate no.	Antibiotic disc				
	AM	GM	CIP	TE	AZ
LB-1	R	R	R	R	R
LB-2	R	R	R	R	R
LB-3	R	R	R	R	R
LB-4	R	R	18 mm (I)	R	R
LB-6	R	R	R	R	R
LB-7	R	R	R	R	R
LB-8	R	R	R	R	R
LB-10	R	R	R	R	R
LB-11	R	R	R	R	R
LB-12	R	R	R	R	R
LB-13	R	R	R	R	R
LB-14	R	R	R	R	R
LB-15	R	R	R	R	R
LB-16	R	R	R	R	R
T-6	R	17 mm (S)	R	R	R
T-7	R	15 mm (S)	R	R	R
T-8	R	14 mm (I)	R	R	R

Note: R = Resistant, S = Sensitive, I = Intermediate, AM = Ampicillin, GM = Gentamycine, CIP = Ciprofloxacin, TE = Tetracycline, AZ = Azithromycin .

Table 3. Antibiogram of isolated *Sterptococcus saliverius* subsp. *thermophiles*

Name of Antibiotics	Disc Conc.	No. of isolate	Sensitivity pattern of *Srteptococcus thermophillus* isolates		
			% R	% I	%S
Ampicillin (AM)	25µg	11	100	-	-
Gentamycine (GM)	10µg	11	100		
Ciprofloxacin (CIP)	5 µg	11	100	-	-
Tetracycline (TE)	30 µg	11	100	-	-
Azithromycin (AZ)	30 µg	11	100	-	-

Table 4. Antibiogram of isolated *Lactobacillus delbruckii* subsp. *bulgaricus*

Name of Antibiotics	Disc Conc.	No. of isolate	Sensitivity pattern of *Lactobacillus bulgaricus* isolates		
			%R	%I	%S
Ampicillin (AM)	25µg	6	100	-	-
Gentamycine (GM)	10µg	6	50	16.33	33.33
Ciprofloxacin (CIP)	5 µg	6	83.33	16.33	-
Tetracycline (TE)	30 µg	6	100	-	-
Azithromycin (AZ)	30 µg	6	100	-	-

In this experiment, yogurt was produced by using direct inoculation of market yogurt (traditional method) (Fig. 4) and also produced by using starter culture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Fig. 5). The associative growth of the two organisms ferment almost all lactose to lactic acid at a rate greater than produced by

either when growing alone (Tarak, 2010). Both organisms produce lactic acid as the main fermentation products. For a proper flavor development, the ratio of *Streptococcus thermophilus* to *Lactobacillus delbrueckii* subsp. *bulgaricus* should be in the range of 1:1 to 3:1 (Pešić-Mikulec and Niketić, 2009).

Samples of yogurt were collected from local market. By using gram staining it was found gram positive rod shaped *Lactobacillus delbruckii* subsp. *bulgaricus* and gram positive spherical to ovoid shaped *Streptococcus saliverius* subsp. *thermophilus.* Isolated bacteria were identified and purified and inoculated a single colony of *Lactobacillus delbruckii* subsp. *bulgaricus* and *Streptococcus saliverius* subsp. *thermophilus* into the milk for making starter culture.

The two bacteria have a mutually growth stimulating effect on one another. Proteolytic enzymes from L. *bulgaricus* break down milk proteins into short peptides and amino acids. These peptides stimulate the growth of *S. thermophilus* which in turn produces formic acid and carbon dioxide which were growth stimulants for *L. bulgaricus.* The associative growth of the two organisms ferments almost all lactose to lactic acid (Irkin and Eren, 2008). This result showed that the pH was acidic and decreased with time which confirms the continuous coversion of lactose to lactic acid by the inoculated starter culture. At the end of the incubation, pH may fall to as low as 4.0. The low pH coagulates the remaining milk proteins, causing the yogurt to thicken (set). This acidic pH prevents the proliferation of other potentially pathogenic bacteria. A temperature of 42^{o}C was maintained for 6-8 hours. After product cooling and storing at 5^{o}C the physical, chemical and microbiological degradation were become slow and fermentation was stopped. Yogurts which are produced by starter cultures have high numbers of yoghurt bacteria means that yogurts produced by using starter cultures have higher therapeutic and/or antimicrobial properties beside of their organoleptic characteristics (Irkin and Eren, 2008). The complex and the specific properties of the yogurt are determined mainly by the biological characteristics of the two species *S. thermophilus* and *Lb. bulgaricus,* their ratio and the biochemical and microbial activities in their mutual development. The interaction between *S. thermophius* and *Lb. bulgaricus* in a yogurt starter culture is described by the ecological term proto-cooperation (Angelov *et al.,* 2009). The positive effect of the protocooperation between the two species is proven by the following characteristic of their mutual metabolism during their cultivation in milk:

- The two bacteria species coagulated separately the sterile milk at temperature 45°C for 6 to 10 h (Figs. 6 and 7). The milk coagulated for 2.0 to 2.5 h when is used a mixed cultures of the two species (Fig. 5),
- The milk coagulated with monocultures of *S. thermophilus* or *Lb. bulgaricus* (Figs. 6 and 7) was with consistency, flavor and aroma different from those of the mixed culture (Fig. 5) coagulated milk that is with thick consistency and well expressed lactic acid flavor and aroma,
- During the mutual fermentation of the two LAB species more volatile aroma compounds (acetaldehyde, diacetylandacetone) were produced and both species showed a higher acid resistance (Fig. 5),
- In case of separate cultivation, *Lb. bulgaricus* and *S. thermophilus* lost faster their typical morphological characteristics and degrade (Fig. 7) while in associated cultivation they kept longer these characteristics (Fig. 5).

Fig. 4. Yogurt production using by traditional method

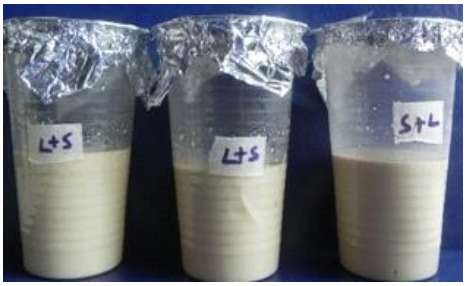

Fig. 5. Yogurt production using starter culture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*

Fig. 6. Yogurt production using only starter culture of *Lactobacillus bulgaricus*

Fig. 7. Yogurt production using only starter culture of *Streptococcus thermophilus*

Conclusion

Lactic acid bacteria is a heterogenous group of bacteria found widely in nature. LABs are added to several probiotic products because of their potential health benefits. Yogurt is a potential source of LAB. A total of 17 isolates were isolated from yogurt samples and finally six species were identified as *Lactobacillus bulgaricus* and eleven species were identified as *Streptococcus thermophilus.* From the experimental results of tested antibiotics showed that all the *Lactobacillus delbruckii* subsp. *bulgaricus* isolates showed resistance to GM (50%), AM (100%), TE (100%), CIP (83.33%), AZ (100%) and all the *Streptococcus saliverius* subsp. *thermophilus* isolates showed 100% resistance to Ampicillin (AM), Gentamycine (GM), Ciprofloxacin (CIP), Tetracycline (TE) and Azithromycin (AZ). Yogurts produced by using startercultures of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* have higher therapeutic and/or antimicrobial properties besides of their organoleptic characteristics. Yogurt is a rich source of protein and calcium, and the fermentation process makes these nutrients easier to absorb. Ongoing studies are revealing many health benefits to this great food, such as boosting immunity and reducing yeast infections and colon cancer. Therefore, the present study suggested potential therapeutic benefit following the consumption of fermented dairy products containing viable lactic acid bacteria (LAB) count and decreased coli form count in the intestine as observed in the analysis.

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