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RESEARCH ARTICLE

EXTENSION OF SHELF LIFE OF CURD – AN INDIAN FERMENTED MILK BY USING A NEW ISOLATE OF *BREVIBACILLUS BREVIS* STRAIN AS STARTER CULTURE

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Abstract
Brevibacillus brevis MMB 12 a new isolated bacteria from curd, was found to set curd within 5 hours of incubation at temperature of 42^{0}C and showed a good shelf life, with thick invertible consistency up to 192 hours (8 days) of fermentation at ambient conditions. Curd was prepared by inoculating *Brevibacillus brevis* MMB 12 strain at 5% level and incubated at temperature of 42^{0}C for 5 hours. The prepared curd was kept at ambient conditions which ranged from 16…24^{0}C throughout the period of experimentation i.e. 8 days. Microbial count ranged between 3.50 ·10^{10} CFU/ml to 2.9·10^9 CFU/ml and the acidity increased from 0.58 to 0.66 % during storage. Curd controled fermentation with a selected strain *Brevibacillus brevis* MMB 12 depicted low acidity compared to the curd inoculated with *Lactobacillus delbrueckii* subsp. *bulgaricus* which was taken as control sample. Mixed curd produced using *Brevibacillus brevis* MMB 12 and *Lb. delbrueckii* subsp. *bulgaricus* curd in 1:1 ratio (v/v) had a shelf life of 72 hours. Flavour, colour, cutting quality and acidity (sourness) were assessed for sensory qualities by 6 specialised trained panelists. Sensory attributes like cutting quality and taste of *Brevibacillus brevis* MMB 12 inoculated curd was rated higher than the other curds. Whereas for colour and flavour the control sample was rated better than *Brevibacillus brevis* MMB 12 inoculated curd.

Keywords: *Brevibacillus brevis*, *Lactobacillus delbrueckii* subsp. *bulgaricus,* multiple culture, curd, shelf life, sensory analysis

Introduction

Milk and dairy products continue to play an important role in the nutrition of people in many parts of the world. Milk has been shown to be ideal for the growth of microorganisms due to its water, protein, carbohydrate, mineral and vitamin contents, all of which are important to many bacteria (Seo *et al.,* 2007; Shan-na Liu *et al.*, 2011). Storage of milk for long periods under refrigeration temperatures has resulted in new quality problems for the dairy industry arising out of the growth and metabolic activities of wild microorganisms at low temperatures (Valerie De Jonghe *et al.*, 2011). To overcome the economical losses fermented milk products came into use. Fermented milks with different names, traditional produced in different countries were reported in prehistoric period and were probably known around the year 2000 BC (Mocanu *et al.,* 2009). Buttermilk and curd were mentioned in Indian Vedas and were known since 800-300 BC (Kansara, 2008).

Curd is an important fermented product used in India as a refreshing beverage. It has mild pleasant flavour, with a clean acidic taste, a creamy white colour with a smooth, gloosy surface and cream layer on top. The mass of curd is expected to be soft and firm, free from gas holes (Srinivasan, 2011). It is well known that curd cannot be kept for more than 24 hours (16 ^{0} ... 24 ^{0}C). On prolonged storage it becomes highly acidic, thus making it unpalatable for human consumption. Therefore

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different methods are applied to restrict the growth of spoilage undesirable microorganisms. Research work at National Dairy Research Institute, Bangalore has shown that bottled curd of long keeping quality can be prepared by using selective strains of lactic acid bacteria (LAB) (Ganguly, 2001). LAB shows remarkable antimicrobial activity against pathogens and spoilage micro-organisms. This is mainly attributed to the production of some compounds with antimicrobial activity as lactic and other organic acids, hydrogen peroxide bacteriocins and other low molecular mass compounds with biopreservative capacity. It has become necessary to identify such cultures, can increase the shelf life of curd traditional produced and preserved under ambient conditions without compromising with the standard quality.

With this background a potential bacterial culture *Brevibacillus brevis* code MMB 12 was isolated from the local samples, which depicted good set curd at ambient conditions within 5 hours.

The present work was undertaken to assess shelf life of curd produced by controled fermentation with *Brevibacillus brevis* MMB12 selected strain. Physical, chemical, microbiological and sensory analysis of the curd were carried out and compared to the quality of the curd prepared using *Lb. delbrueckii* subsp. *bulgaricus*.

Materials and methods

Starter microorganisms

Brevibacillus brevis MMB 12, a new bacterial strain was isolated from a curd sample produced in artisanal conditions by serial dilution method, plated and cultivation in Man Rogosa Sharpe medium (MRS) at temperature of 42 ± 2 ^{0}C, in aerobe conditions (De Man *et al.*, 1960). Upon biochemical characterization isolated strain coded MMB 12 was identified as *Brevibacillus brevis* and stored in Microbial type culture collection (MTCC) with code number 7518 at IMTECH Chandigar, India. *Lactobacillus delbrueckii* subsp. *bulgaricus* was procured from National Dairy Research Institute (NDRI) Bangalore and used as standard strain for all experiments and was maintained on skim milk medium containing 100 g skim milk powder and 15g/l agar in distilled water.

Curd as starter inoculum preparation and characterization

A loopful of freshly 24 hours subcultured of *Brevibacillus brevis* MMB 12 and *Lb. delbrueckii* subsp. *bulgaricus* cultures were inoculated into 10 ml pasteurized milk, incubated at temperature of 42 ± 2 ^{0}C, till curd was produced. This curd with a cell concentration of $5 \cdot 10^8$ CFU/ml was used as preinoculum. Five percent of the preinoculum was aseptically transferred into pasturised milk, and incubated for 4.5 -5.0 hours at temperature of 42 ± 2^{0}C. A mixed starter culture was obtained by mix in 1:1 ratio (v/v) of *Brevibacillus brevis* MMB 12 curd and *Lb. delbrueckii* subsp. *bulgaricus*.

Microbial count was assessed by diluting the samples in saline (0.8 %), and appropriate dilutions were plated on MRS media according to standard methods for the examination of dairy products (AOAC, 2005). The plates were incubated in aerobic condidiotns, for 24 hours, at temperature of 42 ± 2 ^{0}C, and colony counts were recorded.

Curds quality analysis

Acidity was determined as titrable acidity according to the methods of AOAC (2005).

Sensory analysis was carried out for all the curd samples to evaluate the taste and acceptance by a panel of trained judges (all of them were familiar with the product and were using curd in their daily diet). Sensory evaluation was carried out at room temperature in the booth room illuminated by fluorescent lights and the light level was at 70-80 ft-c, which was equivalent to daylight illumination.

A group of six panelists were trained over three sessions for descriptive sensory analysis. The members were familiarized with sensory analysis technique. The common descriptors selected by atleast one third of the panel and few other descriptors sited in literature were utilized in the development of score card (McWilliams, 2001). Panelists were asked to score for flavour, colour, cutting quality, sourness, taste and overall acceptance of curd on a 10 - point hedonic scale. The mean intensity scores of all the attributes (10 =

highly acceptable; 2 = Not acceptable) were calculated and plotted.

Results and discussion

Pasteurized milk inoculated with *Brevibacillus brevis* MMB 12 and *Lb. delbrueckii* subsp. *bulgaricus* and their combinations (1:1 ratio (v/v)) were incubated at 42 ± 2 ^{0}C. Earlier Ledenbach and Marshall (2009) reported 24^{0}C was suitable for rapid growth of spoilage microorganisms, as a result of selected lactic acid bacteria action, acid production conducted at a rapid lowering the pH and the stability of the product was substantially improved. In the present experiment higher temperature of 42 ± 2 ^{0}C were used for incubation, as these were found to be optimum for the starters used to control the lactic fermentation.

Changes in acidity and microbial evolution

The characteristics of the three curd samples produced with *Brevibacillus brevis* MMB 12, *Lb. delbrueckii* subsp. *bulgaricus*, and mixed starter were analyzed to evaluate the dinamic of acidity and lactic acid bacteria cultures. Acidity increased in all the three samples during to 24 from 192 hours of fermentation (Table 1).

Table 1. *Changes during storage of characteristics of curd obtained by using selected Brevibacterium spp. and Lactobacillus spp. fermentative strains*

Time of fermentation (hours)	Acidity (% lactic acid)			Microbial count (10^{10} CFU/ ml)		
	MB	LB	Mix	MB	LB	Mix
24	0.58	0.98	0.68	3.50	0.22	0.56
48	0.63	1.03	0.70	3.65	0.37	0.47
72	0.64	1.26	0.72	4.40	0.50	0.38
96	0.65	1.68	*	2.10	0.36	*
120	0.65	*		1.35	*	
144	0.66			0.82		
168	0.66			0.53		
192	0.66			0.29		

MB – fermentation with *Brevibacillus brevis* MMB 12
LB – fermentation with *Lb. delbrueckii* subsp. *bulgaricus*
Mix – fermentation with mixed starter 1:1 ratio of *Brevibacillus brevis* MMB 12 and *Lb. delbrueckii* subsp. *bulgaricus*

Sample fermented with *Brevibacillus brevis* MMB 12 has lower percentage acidity (0.58 – 0.66%) than the other two samples in which the *Lb. delbrueckii* subsp. *bulgaricus* was present. The curd inoculated with *Lb. delbrueckii* subsp. *bulgaricus* exhibited maximum acidity of 1.68%, after 96 hours of storage. The results of high acid production for *Lb. delbrueckii* subsp. *bulgaricus* culture agreed with the findings of Kamruzzaman *et al*. (2002). El Zubeir *et al.* (2007) reported that when acid production increased the quality of dairy products is affected based on sensorial charactersitics due to sour taste and gas formations. Johanson and Alford (1987) indicated that standard curd should have acidity values in the range of 0.70 % to 0.90 %. But the percentage acidity of *Brevibacillus brevis* MMB 12 curd was lower than that of the standard curd (Table 1). *Brevibacillus brevis* MMB 12 does not have the ability to ferment lactose, hence, the culture did not develop acidity. Acidity developed in this curd is present maybe due to the other spoilage bacteria.

Microbial count of the *Brevibacillus brevis* MMB 12 in curd was very high (Table 1), when compared to other two samples. After 24 hours of fermentation the level of *Brevibacillus brevis* MMB 12 in curd was $3.5 \cdot 10^{10}$ CFU/ml, which increased till 72 hours, then gradually decreased. Earlier researchers have found that the population of viable yoghurt organism increased initially after the manufacture and decreased during a long storage period (Damin *et al.*, 2008).

Mixed cultured curd sample reached a microbial count of $5.6 \cdot 10^9$ CFU/ml, which decreased gradually during storage. But the level of multiplication of *Lb. delbrueckii* subsp. *bulgaricus* in curd was very low when compared to the other

curd samples. The microbial count depicts the viability of the culture in the curd and the decrease in the microbial count could be explained on the basis of reduction in nutrients and accumulation of toxic metabolites (Shemesh *et al.*, 2008). The incubation temperature, dissolved oxygen, lactic and acetic acid content in the product, fermentation time, storage temperature are also presumed to affect the viability of probiotic bacteria in dairy foods (Akin and Guler-Akin, 2005). A quantitative standard bacteria vary from 10^6-10^7 CFU/g viable cells as minimum requirements in probiotic products (Damin *et al*., 2008).

Shelf life and sensory evaluation of fermented products

It is well known that curd cannot be stored for long time due to spoilage produced by of undesirable microorganisms. Therefore the fermentaion with selected starter cultures are applied to restrict the growth of undesirable microorganisms. The curd set with *Brevibacillus brevis* MMB 12 culture when stored at 16…24 °C had a good consistency, without any wheying off till 8 days of storage. This may be due to the metabolites compounds produced during fermentation by strain *Brevibacillus brevis* MMB 12, which have a biopreservative action and thereby increased the shelf life of fermented product. The rate of acidification depends on amount and type of starter culture, thus affecting the sequence of gelation and determining the characteristics of casein matrix (Fadela *et al.*, 2009).

The results on sensory analysis depicts that the curd obtained by fermentation with *Brevibacillus brevis* MMB 12 retained its characters upto 192 hours (Table 2) whereas, the curd obtained by fermentation with *Lb. delbrueckii* subsp. *bulgaricus* has shelf life upto 96 hours (4 days). The curd obtained by using the mixed starters lost its quality after only 72 hours (3 days). This indicates that strains *Brevibacillus brevis* MMB 12 and *Lb. delbrueckii* subsp. *bulgaricus* do not have good syngestic effects.

Table 2. *Sensory analysis of curd obtained by fermentation with selected Brevibacterium brevis and Lactobacillus delbrueckii subsp. bulgaricus fermentative strains*

Time of fermen-tation (hours)	Flavour			Colour			Cutting Quality			Sourness			Taste		
	MB	LB	MIX	MB	LB	MIX	MB	LB	MIX	MB	LB	MIX	MB	LB	MIX
24	7.0	7.0	7.4	8.0	8.2	8.1	8.0	5.0	6.0	7.0	7.5	8.1	7.4	6.1	6.2
48	7.0	7.0	7.0	8.1	8.1	8.1	6.0	5.2	5.8	6.0	7.0	6.7	7.5	6.0	6.0
72	7.0	6.0	7.0	8.0	8.1	8.0	6.7	5.3	5.8	6.1	6.3	6.6	7.4	5.2	6.1
96	7.0	6.0	*	8.0	8.2	*	6.6	5.3	*	6.0	6.3	*	7.4	5.1	*
120	7.0	*	-	8.1	*	-	6.8	*	-	6.1	*	-	7.5	*	-
144	7.0	-	-	8.0	-	-	6.7	-	-	6.0	-	-	7.0	-	-
168	7.1	-	-	8.1	-	-	6.7	-	-	6.1	-	-	6.8	-	-
192	7.1	-	-	8.0	-	-	6.7	-	-	6.1	-	-	6.8	-	-

Samples were stored at room temperature for192 hours. The analysis was conducted based on ten point hedonic scale. Data are average of three trials
MB – fermentation with *Brevibacillus brevis* MMB 12
LB – fermentation with *Lb. delbrueckii* subsp. *Bulgaricus*
Mix – fermentation with mixed starter 1:1 ratio of *Brevibacillus brevis* MMB 12 and *Lb. delbrueckii* subsp. *bulgaricus*

Flavour of *Brevibacillus brevis* MMB 12 fermented product scored 7.0 to 7.1 between 24 to 192 hours of preservation, and *Lb. delbrueckii* subsp. *bulgaricus* curd had maximum score of 7.0, which decreased to 6.0 after of 96 hours. The scoring for the fermented product obtained by mixed cultures was comparatively higher than the other two samples but this sample had a shorter shelf life than the others obtained by fermentation with a single starter cultures. Chemical state of milk proteins and products of their breakdown impart characteristic physical, chemical and sensory properties to most of the dairy products (Sikorski *et al.*, 2001). The flavour of milk product

is therefore a combination of aroma, taste and mouth feel (Gallardo-Escamilla *et al*., 2007). Flavour development occurs due to the release of small peptides and amino acids by proteolysis activity (Liu *et al.*, 2010), or due to lipolysis (Casaburi *et al.*, 2008).

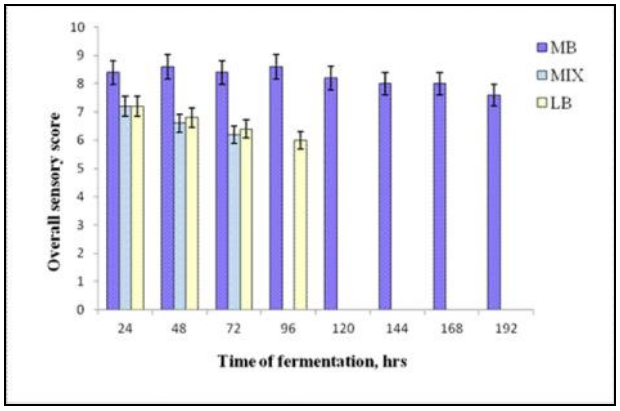

Figure 1. *Overall sensory score of curd obtained by fermentation with selected Brevibacterium brevis and Lb. delbrueckii subsp. bulgaricus fermentative strains MB – fermentation with Brevibacillus brevis MMB 12; LB – fermentation with Lb. delbrueckii subsp. bulgaricus; Mix – fermentation with mixed starter 1:1 ratio of Brevibacillus brevis MMB 12 and Lb. delbrueckii subsp. bulgaricus*

The colour or the appearance of the dairy product plays a vital role in consumption. A pleasant and characteristic appearance adds as one of the sensory character in the product analysis. In the present experiment a better scoring was observed in all the thee samples (Table 2), with maximum scoring of 8.2 for fermented product obtained with *Lb. delbrueckii* subsp. *bulgaricus* with slight variations the colour seems to be pleasant in all the three samples till the shelf life was maintained. The undesirable colour of the product can be observed when the fermented product has variation in the colour due to contamination of spoilage various microorganisms like *Pseudomonas* spps, *Serratia marcesces* and *Rhodotorula glutanis* (Seo *et al.*, 2007).

The cutting quality of fermented product obtained with *Brevibacillus brevis* MMB 12 curd was higher than the other two curd samples, with a maximum score of 8.0 at 24 hours, which varied from 6.0 to 6.7, however, *Lb. delbrueckii* subsp. *bulgaricus* curd had a poor cutting quality than the mixed sample, scores varying between 5.0 to 5.3 and 6.0 to 5.8 respectively. The consistency of all the three curd samples, where *Brevibacillus brevis* MMB 12 depicted a thick invertible consistency till spoilage (192 hours) up to 8 days depicting the firmness of the curd.

Lb. delbrueckii subsp. *bulgaricus* curd had no invertible consistency even at 24 hours, but the mixed curd had a thick invertible consistency till spoilage (72 hours) upto 3 days. Texture results from a complex interaction between milk proteins, acids and exopolysaccharides (Delorme, 2008). Work on exopolysaccharide production by *Brevibacillus brevis* MMB 12 strain is yet to be initiated. The syneresis of the kept curd samples was evaluated. No whey off was observed in the curd obtained by fermentation with *Brevibacillus brevis* MMB 12 up till of 192 hours (8 days of preservation). The carboxyl and amine groups that are liberated during proteolysis cause a decrease in water activity by binding water molecules (McSweeney, 2004). Because the strain *Brevibacillus brevis* MMB 12 is a lactose negative bacterium its metabolic pathway may be directed towards the utilization of proteins and lipids present in the milk. Whey off was observed after 48 hours and 72 hours respectively in curds obtained with *Lb. delbrueckii* subsp. *bulgaricus* or by using mixed strater cultures.

The sourness is the result of the percentage acidity in the curd samples. The sensory score for sourness of mixed sample was higher than product fermented with *Brevibacillus brevis* MMB 12 or *Lb. delbrueckii* subsp. *bulgaricus* curd, scoring 8.1, 7.0 and 7.5 respectively (Table 2). After 24 hours of preservation the sensory score for sourness of *Brevibacillus brevis* MMB 12 curd reduced and varied from 6.0 to 6.1, but the scores of other two samples was higher than the *Brevibacillus brevis* MMB 12 curd. The sensory score for taste of the *Brevibacillus brevis* MMB 12 curd ranged between 7.0 - 7.5 from 24 hours to 120 hours of preservation, after which the scores slightly decreased to 6.8. The sensory score for taste of the *Lb. delbrueckii* subsp. *bulgaricus* curd and mixed curd was very low when compared to *Brevibacillus brevis* MMB 12 curd. *Lb. delbrueckii* subsp. *bulgaricus* curd and mixed curd had a sensory

score of 6.1 and 6.2 at 24 hours of preservation respectively. The least score of *Lb. delbrueckii* subsp. *bulgaricus* curd was at 96 hours (5.1) and 6.1 for mixed curd by 72 hours, after which the samples were spoilt.

The overall sensory score of *Brevibacillus brevis* MMB 12 was high throughout the studies i.e. 8 days which ranged from 7.6 to 8.6 as shown in Figure 1. The overall sensory score of the mixed curd sample reduced from 7.2 to 6.2 as the time of storage of the curd increased. Its shelf life was maintained till 72 hours (3 days). However the score of *Lb.delbrueckii* subsp. *bulgaricus* curd was very low at 24 hours, which increased marginally after of 48 hours of storage. Based on these data it is clear that the *Brevibacillus brevis* MMB 12 curd had a better preference when compared to the other two samples along with shelf life upto 8 days at room temperature (16…24°C).

Curd obtained by fermentation with *Brevibacillus brevis* MMB 12 had a specific flavour, texture, sourness and overall acceptability with a long shelf life up to 8 days.

Conclusions

Traditional fermented milk based products are always fermented with a mixed consortium of organismns. The requeirement to select isolate and study the characters for safe and accaptable products is always a challenge. In the present study *Brevibacillus brevis* MMB 12 strain seems to be a promising candidate for production of safe traditional fermented milk product by increasing the stability and sensorial characteristics compared with artisanal products obtained by fermentation with *Lb. delbrueckii* subsp. *bulgaricus*.

The product developed a very good shelf life compared to he traditional product, due to its low acidity, pleasing aroma and acceptable sensory characters. As the culture is a lactose negative culture the curdling is mainly due to the action of the proteolytic and lypolytic enzymes. Further investigations on the enzyme production and the characters of the product needs to be investigated. Such a study would lead to formulation of fermented dairy products based on a nutritional requirement of certain population.

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