Effect of bacterial exopolysaccharide on the physical properties of acid milk curd by lactic acid fermentation

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Abstract. Exopolysaccharide (EPS) is an extracellular slime polysaccharide from bacteria and one of EPS produced by Alcaligenes faecalis var. myxogenes strain 10C3 which found by Harada for the first time in 1965. In this study, it was used Lactobacillus delbrueckii subsp. bulgaricus B-5b, which obtained from Japan Milk Product Technology Association, Tokyo, Japan. During the course of the investigation, the culture was routinely propagated in 10% RSM. The RSM was added with 0, 0.5 and 1% of EPS, autoclaved at 121°C for 15 min and tempered to 37°C prior to inoculation. A 0.1% inoculum was added to the RSM and the culture was allowed to incubate at 37°C overnight. Milk curd investigated toward growth curve of cell bacteria numbers, pH, acidity, viscosity, rheometric properties. The result indicated that increasing EPS concentration cause of increases the pH and the pH of milk increases its viscosity perhaps by swelling of casein micelles, but a more drastic pH decrease causes the viscosity to increase, which is caused by aggregation of casein. On the other side, increasing the EPS concentration decreases acidity as expected. It is well established that milk which has been heated at a temperature above 70°C has a longer coagulation time and forms a weaker curd than the original unheated milk.

1. Introduction

There have been many investigations involving optimization of milk curd texture. These studies have demonstrated that the total solid and fat level in the milk, heat treatment of the milk prior to inoculation, homogenization, incubation conditions, and handling of the ripened coagulum will all affect the body of the final milk product. Another major way to affect the body yogurt is through the addition of stabilizers such as gelatin, pectin or another polysaccharide. Stabilizers are added to the product to increase viscosity as well as to decrease susceptibility to syneresis [1].

In the dairy industry, the use of polysaccharide producing bacteria is of interest with respect to the improvement of body and texture of yogurt, in particular in France and the Netherland where the addition of plant or animal stabilizers is prohibited.

Extracellular slime polysaccharide of Alcaligenes faeicalis var. myxogenes strain 10C3 was found by Harada for the first time in 1965. Harada was isolated, purified this polysaccharide, and concluded that the EPS contained about 10% succinic acid, glucose 70-80%, and a small amount of galactose and mannose. It seems to have β-glycosidic linkages [2]. This EPS is added to food to improve their properties and also to make new foods [3]. Microbial exopolysaccharides often show clearly identified...
properties that form the basis for a wide range of applications in food as industrial thickeners, gels and stabilizers [4,5], as antitumor [6].

Lactic acid bacteria are organisms which ferment sugar to yield lactic acid. The consumption of a minimum viable cell number of $1.0 \times 10^7$ to $1 \times 10^9$ cells per day are necessary to have any chance of developing beneficial effect in human. Cultured milk products always have been an important part of the human diet. They often are credited with various therapeutic or prophylactic value. Microflora of cultured milk may vary according to the area of origin and method of preparation [7]. *Lb. delbrueckii* subsp. *bulgaricus* is a homo fermentative organisms that produce principally lactic acid from sugar [8]. It was originally isolated by Grigoroff from fermented milk. It received considerable attention as a result of its recommendation to control intestinal putrefaction, what was believed as an important factor in limiting the length of life in man. The organisms stain readily and are Gram-positive. They grow especially well in milk and can stand higher acidities such as a pH range from 3 to 4 [9]. A comparatively high temperature, such as 32 to 38°C, greatly favors growth [10].

Although polysaccharide has been widely investigated, little information exists on their effect polysaccharide in reconstituted skim milk (RSM) fermented by lactic acid bacteria. This study examined the effect of EPS on the physical properties of acid milk curd by lactic acid fermentation.

2. Materials and Methods

2.1 Lactic Acid Bacteria Cultures

In this study, it was used *Lactobacillus delbrueckii* subsp. *bulgaricus* B-5b, which obtained from the laboratory of animal product technology, Hasanuddin University. During the course of the investigation the culture was routinely propagated in 10% Reconstituted Skim Milk (RSM). The RSM was autoclaved at 121°C for 15 min and tempered to 37°C prior to inoculation. A 0.1% inoculum was added to the RSM and the culture was allowed to incubate at 37°C overnight.

The ability of *Lb. Delbrueckii* subsp. *bulgaricus* B-5b to grow from 0 -80 h was determined. A 1% starter culture was inoculated into 5 tubes containing 10 ml of 10 % of RSM. The criteria to determine growth were viscosity, pH, % lactic acid, bacteria numbers (CFU/ml) and milk coagulation.

2.2 Preparation of acid milk curd

Acid milk curd sample was made from 10, 15 and 20% of RSM. EPS (produced by *Lactobacillus bulgaricus*) was added in different concentration (0, 0.2, 0.4, 0.6, 0.8, 1.0%), heated at 60°C for 30 min and 85°C for 30 sec., cooled to 37°C, inoculated with 1% (v/v) *Lb. delbrueckii* subsp. *bulgaricus* B-5b, and incubated at 37°C for 16 hours.

2.3 Cell bacteria numbers

One ml of the *Lb. delbrueckii* subsp *bulgaricus* B-5b cultures in 10% RSM added to 9 ml of sterile distilled water and thoroughly mixed to make a 1/10 dilution, from which decimal dilutions were prepared (1/100, 1/1000, and so on). The standard plate count method was applied. From each of the 0.1 ml previously prepared, dilutions were plated, in duplicates, using brom-chresol purple agar medium (BCP). Inoculated plates were incubated at 37°C for 2 days. Plates showing 30-300 colonies were counted using a digital colony counter (DC3 Model, Japan). The total colony count per ml rinse was calculated and the colony forming unit/ml was recorded according to Marshall [11]. The samples were taken and analyzed at incubation time from 0 -80 h.

2.4 Titratable acidity

Cultures were made in 10% RSM, heated at 60°C for 30 min, inoculated with *Lb. delbrueckii* subsp. *bulgaricus* B-5b, and incubated 37°C at different incubation times. Two grams of this cultures were added 4 ml distilled water, added 0.5 ml of phenolphthalein indicator and titrated with 0.1 M sodium hydroxide to the first permanent color change to pink.
The growth curve of acidity was calculated according to the following formula;

\[
\text{Acidity (\%)} = \frac{\text{ml NaOH} \times N \text{NaOH} \times 9}{\text{Weight of sample}} \quad [11]
\]

Or

\[
\text{Acidity (\%)} = \frac{\text{ml NaOH} \times N \text{NaOH} \times F \times 9}{\text{Weight of sample (g)}}
\]

Where, \( F \) = Factor of NaOH solution
Acidity is expressed as the percentage of lactic acid. One ml of 0.1 N sodium hydroxide (NaOH) = 0.009 gr of lactic acid.

2.5 pH
The pH value was measured by using a TOA HM-30 pH meter. All of the pH measurement methods.

2.6 Viscosity
The viscosity was studied by using a viscometer (Tokimec Inc, Visconic ED-Model). Experimental conditions were steady shear rate of 1-100/s along with MK 50 rotor assembly and NV sensor system operating at 25°C.

2.7 Effect of heat treatment
EPS (1%) was added to 10% RSM and then heated at 60°C for 30 min and 85°C for 30 sec. Incubation was done at 37°C for 16 h. The acid milk curd formed were investigated for viscosity, pH, acidity, and rheological properties.

2.8 Effect of milk solid content
Reconstituted skim milk 10, 15 and 20% was added with 1% of EPS and then heated at 60°C for 30 min and 85°C for 30 sec, incubated at 37°C for 16 h. The acid milk curd was investigated toward physical properties.

2.9 The growth of cell number of culture in EPS-added acid milk curd
One ml of the acid milk curd containing 0, 0.5 and 1.0% of EPS added to 9 ml of sterile distilled water and thoroughly mixed to make a dilution 1/10, from which decimal dilutions were prepared (1/100, 1/1000, and so on), and the standard plate count method was applied. From each of the previously prepared dilutions, 0.1% was plated, in duplicate, using bromochresol purple agar (BCP). Inoculated plates were incubated at 37°C for 2 days. Plates showing 30-300 colonies were counted using a digital colony counter (DC3 Model, Japan). The colony count per ml was calculated and the colony forming unit/ml was recorded. The samples were taken and analyzed at incubation time from 0 h to 16 h.

3. Results and Discussions

3.1 Effect of curdlan on Total Plate Count of Lactic Acid Bacteria of Lb. bulgaricus
The effect of incubation temperature on growth tests of \( \text{Lb. delbrueckii} \) subsp. \( \text{bulgaricus} \) B-5b is shown in table 1. This strain had an optimal growth temperature at 37°C with pH isoelectric point (about 4.5) in the 10% RSM. When stored at room temperature (25°C) the cell number of \( \text{Lb. delbrueckii} \) subsp. \( \text{bulgaricus} \) in 8, 10 and 12% autoclaved RSM were not significant, and lactic acid production was lower than storing at 30 or 37°C. The kinetic of lactic acid production is partially growth linked, with lactic acid continuously being produced at a low rate as long as a carbon source is available, even when cell growth has increased.
As shown in Table 2, acid milk curd (10% RSM) at incubation temperature 25°C, 30°C, 37°C, the pH was decreased to 4.84, 4.02, 3.75, respectively after 36 h of incubation. When incubation took place at 25°C, pH values decreased linearly to 3.9 until 54 h incubation and did not change until 90 h of incubation. However, when incubation took place at 37°C, the pH isoelectric point was reached after 18 h of incubation time. The final pH appears to play an important role in determining the stability of lactic acid bacteria in fermented milk products. Higher-culture pH values allow a longer survival of *Lactobacillus bulgaricus*. Hence, when the pH of milk cultures was maintained at near neutrality, the viable cell count of the milk cultures was maintained constant for longer than a month at 25°C. As shown in Table 2, viable cell counts were maintained constant for 54 until 90 h at an incubation temperature of 30°C. However, in an incubation temperature of 37°C, cell viable numbers were decreased after 54 h of incubation time.

According to the commercial potential of these cultures it is useful to know their growth characteristics. Thus, the growth curve of *Lb. delbrueckii* subsp. *bulgaricus* B-5b shows that in 10% RSM most cell growth occur during the first 8 - 14 h of incubation time at 37°C. The growth curve of acidity was increased linearly until 16 h of incubation time, whereas, the pH was decreased linearly until 16 h of incubation time. Viscosity development stays constant for the 0 - 6 h of incubation time and increased up from 8 - 16 h of incubation time.

### Table 1. Effect of incubation temperature and acid milk solid content of *Lb. delbrueckii* subsp. *bulgaricus* B-5b on viscosity, acidity, pH and cell bacteria numbers

| Item           | 8% skim milk | 10% skim milk | 12% skim milk |
|----------------|--------------|---------------|---------------|
| Viscosity      | 0.35         | 0.77          | 8.12          |
| Acidity        | 0.22         | 0.32          | 0.73          |
| pH             | 5.49         | 5.41          | 4.99          |
| Cell number    | 2.08x10⁶     | 3.64x10⁶      | 3.56x10⁶      |

3.2 Effect of EPS on pH, acidity, and viscosity of acid milk curd

According to the commercial potential of these cultures it is useful to know their growth characteristics. Thus, the growth curve of *Lb. delbrueckii* subsp. *Bulgaricus* B-5b shows that in 10% RSM most cell growth occur during the first 0 - 5 h of incubation time at 37°C (fig. 1). As shown in figure 2 acid milk curd (10% RSM) with addition 0, 0.5 and 1 % of EPS at incubation time from 0 to 80 h, the pH was decreased to 4.84, 4.02, 3.75, of incubation. Increasing EPS concentration increases the pH and the pH of the milk product increases its viscosity perhaps by swelling of casein micelles, but a more drastic pH decreases causes the viscosity to increases, which is caused by aggregation of casein. On the other side, increasing the EPS concentration decreases acidity as expected. It is well established that milk which has been heated at temperature above 70°C, has a longer coagulation time and forms a weaker curd than the original unheated milk [12]. When milk was heated from 25 - 50°C and 75°C for few minutes, a reduction in pH was observed [13]. An increase of viscosity during the course of coagulation indicates the formation aggregates.

The plots of viscosity as a function of EPS concentration for 10, 15, and 20% acid milk curd preheated at 60°C and 85°C. However, the acid milk curd heated at 85°C exhibited a greater apparent viscosity by acid milk curd heated at 60°C. An increased rigidity of milk coagulated at higher temperatures was believed to be due to the effect of acid on protein structure at the higher temperature. It would appear that the rate of acid production also affects gel firmness [14]. The apparent viscosity for both preheating at 60°C and 85°C increases with increasing EPS concentration. However, the 20% skim milk of acid milk curd showed the greatest viscosity than 15% or 10% of RSM. The increasing viscosity may be due to the fact that some casein, particularly B-casein, start to dissociate from the
micelles, and dissolved casein molecules have a higher hydrodynamic volume. Consequently, the viscosity rises [15].

Table 2. Effect of solids content, incubation temperature and incubation time of autoclaved reconstituted skim milk fermented by *Lb. delbrueckii* subsp. *bulgaricus* B-5b on viscosity, acidity, pH and bacteria cell numbers

| Milk solid content (%) | Incubation temperature (°C) | Incubation time (hours) | Viscosity (mpa/sec) | Acidity (%) | pH | Bacterial count (CFU/ml) |
|------------------------|-----------------------------|-------------------------|---------------------|-------------|----|-------------------------|
|                        | 0                           | 18                      | 36                  | 54          | 72 | 90                      |
| 8                      | 25                          | 0.37                    | 0.39                | 3.08        | 40.2| 42.6                    | 46.0 |
| 10                     | 25                          | 0.38                    | 0.43                | 14.22       | 50.4| 57.4                    | 59.0 |
| 12                     | 25                          | 0.43                    | 0.55                | 14.24       | 92.2| 122.7                   | 152.3|
| 8                      | 30                          | 0.46                    | 0.77                | 14.19       | 48.9| 49.0                    | 131.4|
| 10                     | 30                          | 0.49                    | 0.96                | 16.20       | 52.7| 93.0                    | 158.6|
| 12                     | 30                          | 0.68                    | 0.99                | 20.54       | 135.7| 217.0                   | 274.0|
| 8                      | 37                          | 0.61                    | 8.12                | 15.47       | 53.8| 153.0                   | 185.0|
| 10                     | 37                          | 0.86                    | 8.79                | 17.20       | 76.2| 97.8                    | 168.0|
| 12                     | 37                          | 1.00                    | 8.87                | 18.43       | 167.76| 220.4                   | 249.8|
| 8                      | 25                          | 0.15                    | 0.22                | 0.47        | 0.77| 0.97                    | 1.16 |
| 10                     | 25                          | 0.17                    | 0.23                | 0.57        | 1.05| 1.19                    | 1.23 |
| 12                     | 25                          | 0.25                    | 0.32                | 0.71        | 1.15| 1.31                    | 1.30 |
| 8                      | 30                          | 0.15                    | 0.32                | 0.86        | 1.17| 1.18                    | 1.45 |
| 10                     | 30                          | 0.17                    | 0.49                | 1.09        | 1.40| 1.40                    | 1.62 |
| 12                     | 30                          | 0.22                    | 0.40                | 1.19        | 1.52| 1.61                    | 1.68 |
| 8                      | 37                          | 0.14                    | 0.73                | 1.15        | 1.38| 1.20                    | 1.42 |
| 10                     | 37                          | 0.20                    | 0.77                | 1.27        | 1.52| 1.69                    | 1.71 |
| 12                     | 37                          | 0.24                    | 0.80                | 1.57        | 1.76| 1.77                    | 1.81 |
| 8                      | 25                          | 6.51                    | 5.49                | 4.85        | 3.97| 3.78                    | 3.81 |
| 10                     | 25                          | 6.43                    | 5.42                | 4.84        | 3.90| 3.87                    | 3.84 |
| 12                     | 25                          | 6.33                    | 5.40                | 4.78        | 3.85| 3.80                    | 3.78 |
| 8                      | 30                          | 6.50                    | 5.41                | 4.02        | 3.71| 3.78                    | 3.63 |
| 10                     | 30                          | 6.42                    | 5.36                | 4.00        | 3.70| 3.78                    | 3.63 |
| 12                     | 30                          | 6.32                    | 5.24                | 4.04        | 3.78| 3.57                    | 3.33 |
| 8                      | 37                          | 6.51                    | 4.99                | 3.75        | 3.61| 3.78                    | 3.36 |
| 10                     | 37                          | 6.44                    | 4.83                | 3.75        | 3.60| 3.58                    | 3.36 |
| 12                     | 37                          | 6.34                    | 4.15                | 3.77        | 3.59| 3.43                    | 3.30 |

Viscosity (η) is defined as the ratio of shearing stress (τ) to shear rate. Viscosity usually is well described by the semi-empirical Eilerequation: \( \eta = \eta_0 (1 + 1.25 \phi/1 - \phi/\phi_{max})^2 \). This equation predicts fairly well the viscosity of skim milk, cream, concentrated milk, and milk ultrafiltrate up to \( \phi=0.6 \) [15].

There are considerable differences in viscosity among lots of milk, and the hydrodynamic volume of the casein micelles must be an important variable. The content of calcium phosphate in the micelles...
and Ca\textsuperscript{2+} activity play an important part [15]. Viscosity measurement is difficult to interpret, because they may be affected not only by polysaccharides released by bacteria but also by the type of the polymer and the effects of the metabolic products which are excreted in the medium [16, 17].

![Figure 1](image1.png)

**Figure 1.** Effect of incubation time (h) on growth tests of *Lb. delbrueckii* subsp. *bulgaricus* B-5b in acid milk curd by addition 0, 0.5 and 1% of curdlan.

![Figure 2](image2.png)

**Figure 2.** The effect of EPS concentration on pH of acid milk curd fermented by *Lb. delbrueckii* subsp. *bulgaricus*.

Increasing EPS concentration increases the pH and the pH of milk increases its viscosity perhaps by swelling of casein micelles, but a more drastic pH decreases causes the viscosity to increase, which is caused by aggregation of casein. On the other side, increasing the EPS concentration decreases acidity as expected. It is well established that milk which has been heated at temperature above 70\textdegree C has a longer coagulation time and forms a weaker curd than the original unheated milk. When milk was heated from 25 to 50\textdegree C and 75\textdegree C for few minutes, a reduction in pH was observed [13]. An increase of viscosity during coagulation indicated the formation of micelle aggregates.
References
[1] Schellhaass S M and Morris H A 1985 Rheological and scanning electron microscopic examination of skim milk gels obtained by fermenting with ropy and non-ropy strains of lactic acid bacteria Food Micros. 4 279-283
[2] Takahashi F, Harada T, Koreeda A and Harada A 1986 Structure of curdlan that is resistant to (1→3) β-D-Glucanase Carbohydrate Polymers 6 407-421
[3] Harada T 1992 The story of research into curdlan and the bacteria producing it Trend in Glycoscience and Glycotechnology 4(17) 309-317
[4] Malaka R 1997 Effect of Curdlan, a Bacteria Polysaccharide on the Physical Properties and Microstructure of Acid Milk Curd by Lactic Acid Fermentation Master Thesis (Miyazaki: Faculty of Agriculture Miyazaki University Japan)
[5] Malaka R, T Ohashi and S Baco 2013 Effect of Bacteria Exopolysaccharide on Milk Gel Formation Open J Forest. 3(4B) 10-12
[6] Malaka R, E Abustam and S Baco 2016 Antitumor Activity (In-vitro) of Extracellular Polysaccharide Produced by Ropy Lactobacillus delbrueckii ssp. bulgaricus Isolated from Traidtional Fermented Milk Int. J. Chemist. Pharm. Sci. 4(5) 246-249
[7] Macura D and P M Townsley 1984 Scandinavian ropy milk Identification and characterization of endogenous ropy lactic streptococci and their extracellular excretion J. Dairy Sci. 67 735-744
[8] Takao S, S Ibarakura, H Saito, H Takahashi 1989 Appl Microb I
[9] Benno Y 1990 Identification and classification of lactic acid bacteria The Bimonthly J. of Microorg. 6(1) 3-14
[10] Morichi T 1984 Practical Handbook of Manufacture Technology in Dairy Chemistry
[11] Marshall R T 1992 Standar Methods for the Examination of Dairy product (Washington DC: American Public Health Association)
[12] Delgleish DG 1990 The effect of denaturation of β-lactoglobulin on renneting a quantitative study Milchwissenschaft 45(8) 491-494
[13] Sharma S K, A R Hill and H D Goff 1990 The effect of heat treatment of ultrafiltered milk on its coagulation properties Milchwissenschaft 45(7) 432-435
[14] Schellhass S M and Morris H A 1985 Rheological and scanning electron microscopic examination of skim milk gels obtained by fermenting with ropy and non-ropy strains of latic acid bacteria Food Micros. 4 279-287
[15] Walstra P and R Jennses 1987 Dairy Chemistry and Physics (New York: John Wiley and Sons)
[16] Cerning, J 1990 Extracellular polysaccharide produced by lactic acid bacteria FEMS Microbiol Rev 87 113-130.
[17] Bottanzi V and F Bianchi 1986 Types of microcolonies of lactic acid bacteria, formation of void spaces and polysaccharides in yogurt Scienza E Tecnica Lattiero-Casearia 37(4) 297-315