Isolation of exopolysaccharides producing-lactic acid bacteria for fermented milks products

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Abstract

Exopolysaccharides (EPS) produced by lactic acid bacteria (LAB) play an important role in various industries particularly dairy industry. Their usage including improve the viscosity, texture and mouth-feel of the fermented milks. This present study aims to isolate EPS-producing lactic acid bacteria (LAB) from fermented dairy and non-dairy foods for their application in fermented milk products. Among the 107 isolates, 27 isolates were EPS producing bacteria with LAB characteristics (gram positive bacteria with catalase negative). Among these 4 gave positive results for litmus milk test; 2 gave ropy colony (isolates L17 and L20) and another one (L26) with mucoid appearance. L26 gave highest yield of 19.38 g L^{-1} EPS while L20 and L17 gave 16.37 and 15.26 g L^{-1} EPS respectively. In addition, EPS from L17 showed best water holding capacity judging from its syneresis percentage. The preliminary results show tendency for their applications in fermented dairy products.

Keywords: exopolysaccharides, lactic acid bacteria, fermented milks

Introduction

The increasing demand for natural polymers in various industrial applications led to highly interested in microbial polysaccharides. Numerous microorganisms were reported as having ability in synthesizing exopolysaccharides (EPS) or extracellular polysaccharides secreted outside cell wall during their growth. These EPS can exist in two basic forms: as capsule or slime polysaccharides (Kumar et al. 2007). EPS biopolymers consist of long-chain polysaccharides that can further divided into homopolysaccharides and heteropolysaccharides. Homopolysaccharides composes only one type of monosaccharide such as dextran, pullulan, curdlan while heteropolysaccharides composed of more than one types of monosaccharide such as gellan, xanthan, kefiran. Polysaccharides are biothickeners commonly used as food additives as viscosifying, stabilizing, emulsifying or gelling agents.

EPS-producing Lactic acid bacteria (LAB) have alternative way of improving the texture and stability of fermented dairy and non-dairy products. Some properties of LAB such as flavour and texture formation are especially important to the food and feed industries since they can be applied in various products and LAB are food grade organisms due to their GRAS (generally regarded as safe) status for example of genera *Streptococcus*, *Lactobacillus* and *Lactococcus*. EPS produced by LAB have potential as natural additives and also be produced in situ. Incorporation of EPS or EPS-producing (EPS+) cultures in dairy foods can provide viscosifying, stabilizing and water-binding functions. In addition, EPS also contributes the mouth-feel, texture and taste perception of fermented dairy products (Lavanya et al. 2011). The in situ production of EPS are very important in the manufacture of fermented dairy products, such as yogurt, drinking yogurt, cheese, cultured cream and milk-based desserts. Moreover, EPS were produced by LAB have beneficial effects on human health such as cholesterol-lowering, immunomodulation and prebiotic effect.

Most of the EPS-producing LAB can isolated from different fermented foods such as sourdoughs, sausages, table olives, cheeses, yoghurt, kefir, other fermented dairy products and some traditional foods from non-industrialized countries. Furthermore, EPS-producing strains can found other environments such as gut of different animals and humans. Selecting for LAB strains with interesting properties to be used as new, functional starter cultures may lead to improved fermentation process and an enhanced quality of the end product (Leroy and De Vuyst 2004). The aim of this study was to isolate EPS produced by LAB from fermented dairy and non-dairy food for application in fermented milk products.

Methodology

Screening and isolation of EPS-producing LAB

Samples collection

The lactic acid bacteria isolates were obtained from raw milk, fermented foods and fruit juices collected from Bangkok, Nakhon Ratchasima and Nakhon Si Thammarat province. Samples were taken to the laboratory for microbiological analyses.

Screening of EPS-producing LAB

All samples were incubated at 37°C for 24 hours before analysis. From each sample, a 1:10 dilution was subsequently made using 0.1% peptone water followed by making a 10 fold serial dilution and the appropriate dilutions were plated onto Man, Rogosa and Sharpe (MRS) agar with (4% w/v) sucrose via spread plate method and incubated at 30°C for 24 h. Isolates that produced slimy colonies on agar plate were recorded as capable of producing EPS. For every sample, 5 - 6 slimy colonies were randomly selected and purified by following the streaking method on MRS agar (Vijayendra et al. 2008).

Isolation of the EPS-producing LAB for growth in milk

Each individual colony of all EPS-producing isolates on MRS agar was subjected for preliminary characterisation of LAB by gram staining and catalase reaction (Paulo et al. 2012). Isolates that showed gram positive and catalase negative reactions were screened for their abilities to grow in milk by litmus milk test at 30°C for 24 h.

Morphological study

The positive isolates for litmus milk test were streaked on MRS agar with 4% sucrose and incubated at 30°C for 24 h. Then, mucoidy and ropiness of colonies on agar plates were examined by touching colonies with a sterile loop. The ropy colonies are able form a long filament when extended with a loop whereas the mucoid colonies have a slimy appearance on agar plates and were not able to produce strands by this method. (Dierkaen et al. 1997; Ruas-Madiedo and de los Reyes-Gavilan 2005). Gram stain was done on the selected colonies followed by observing their morphological appearance under microscope.

Characterisation of EPS

Inoculum preparation
The EPS producing isolates grew on agar plates and gave positive result for litmus milk test (mucoid, ropy) were selected and further cultured in MRS broth supplemented with 4% sucrose. This was done on a rotary shaker (200 rpm) at 30°C until the OD_{550} reach 0.8-1.0 then a 10% (v/v). This inoculum was further transferred into production medium of MRS broth supplemented with 4% sucrose on a rotary shaker (200 rpm) at 30°C for 24 h.

Isolation of exopolysaccharides
After cultivation, broth cultures were added with trichloroacetic acid solution at final concentration of 4% (w/v), shook and allow to stand for 30 minutes which precipitate of protein and bacterial cells were formed and removed by centrifugation at 8000 rpm for 20 minutes. Supernatant was then mixed with two volume of cold 95% ethanol, kept at 4°C overnight. EPS was recovered by centrifugation at 8000 rpm for 30 minutes, the resulting pellet was dissolved in distilled water and mixed with two volume of cold 95% ethanol, stored at 4°C overnight, and centrifuged at 8000 rpm for 30 minutes to collect the EPS. After 24 h of freezing at -20 °C followed by lyophilization, the dry weight of the EPS obtained was determined. Total sugar content of EPS was determined by phenol-sulfuric acid method using glucose as standard (Dubois et al. 1956). The results were expressed in milligram of glucose per litre while total protein of EPSs assayed by protein dye binding method (Bradford 1976).

Measurement of solubility of EPS
Solubility of EPS was determined in distilled water and solvents including methanol, acetone, isopropanol and n-butanol at room temperature.

Measurement of water-holding capacity
Water-holding capacity of EPS was determined according to the method by Tako et al. (1982) via ascending paper chromatography by preparing 0.5 % (w/v) solution of EPS in distilled water, using guar gum, carrageenan and xanthan gum (food grade) as standards. The end of the filter paper was dipped into 0.5% (w/v) EPS solution for one hour. Rate of syneresis was calculated as percentage of the developed length on filter paper of EPS solution/developed length on filter paper of distilled water.

Results

Isolation for EPS producing LAB

In this study, a total of 107 isolates were obtained from fermented dairy and non-dairy food collected from different sources, 27 isolates were showed tendency of EPS producing bacteria. All of these EPS producing bacteria were of gram positive and exhibited catalase negative as typical characteristics of LAB (Table 1). Four isolates (L17, L20, L26 and L27) of LAB with EPS were able to grow in milk and gave positive results at 24 h as depicted in Figure 1.

Table 1 Morphology of EPS-producing isolates from fermented dairy and non-dairy food

Isolates	Gram strain	Shapes	Catalase test	Litmus milk test
L01	+	Rod	-	-
L02	+	Coccus	-	-
L03	+	Short rod	-	-
L04	+	Short rod	-	-
L05	+	Short rod	-	-
L06	+	Coccus	-	-
L07	+	Coccus	-	-
L08	+	Rod	-	-
L09	+	Rod	-	-
L10	+	Rod	-	-
L11	+	Coccus	-	-
L12	+	Coccus	-	-
L13	+	Rod	-	-
L14	+	Short rod	-	-
L15	+	Short rod	-	-
L16	+	Short rod	-	-
L17	+	Rod	-	+
L18	+	Short rod	-	-
L19	+	Short rod	-	-
L20	+	Short rod	-	+
L21	+	Short rod	-	-
L22	+	Short rod	-	-
L23	+	Rod	-	-
L24	+	Short rod	-	-
L25	+	Short rod	-	-
L26	+	Short rod	-	+
L27	+	Short rod	-	+

+ = positive - = negative

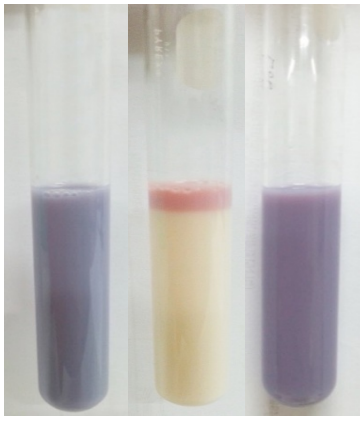

Figure 1 Result of litmus milk test: control (left), positive with the reduction of methylene blue with curd formation by strain L26 (middle) and negative result without methylene blue reduction of strain L1 (right)

Determination of mucoid, ropy colonies

EPS-producing LAB that grew in milk were observed for the mucoidy and ropiness appearance on MRS with 4% (w/v) sucrose agar. After 24 h incubation at 30°C, strain L17 and L20 were able to form ropy colonies as show in Figure 2 whereas strain L26 and L27 form mucoid colonies on agar plates.

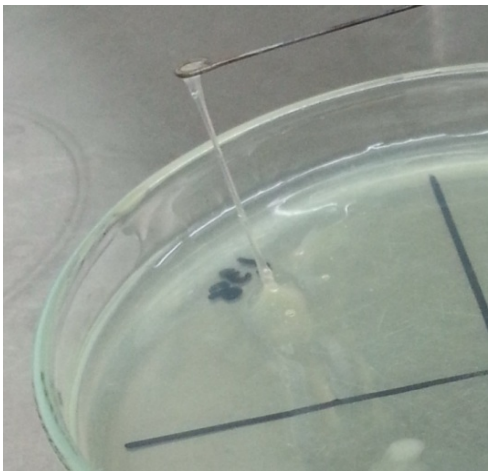

Figure 2 The appearance of ropy strand formed by L17 strains on MRS with 4% (w/v) sucrose agar plates

EPS production

Table 2 showed the EPS produced by the selected 4 strains, the highest amounts of EPS producer was L26 in which approximately 19.38 g $L^{-1}$was obtained. The total carbohydrate and proteins content of EPSs produced by all strains are presented in Table 2.

Table 2 EPS production, total carbohydrate and total protein contents of EPS

EPS from strains	Yield ($g\ L^{-1}$)	Total carbohydrate ($\mu g\ mL^{-1}$)	Total protein ($\mu g\ mL^{-1}$)
L17	15.26	112.94	48.00
L20	16.37	95.96	24.14
L26	19.38	111.34	26.57
L27	3.42	102.51	32.29

Solubility of EPS

The EPS from strain L17, L20, L26 and L27 were partially soluble in water at room temperature while insoluble in all organic solvents tested (methanol, acetone, isopropanol and n-butanol).

Water-holding capacity

Water-holding capacity in term of rate of syneresis (%), the syneresis rate of 0.5% polysaccharide solutions (L17, L20, L26, L27, xanthan gum, guar gum and carrageenan) are showed in Table 3. Rate of syneresis of all polysaccharide are rather high compared with the control polysaccharides, indicated lower water-holding capacity since percentage of syneresis is the invert of water-holding capacity.

Table 3 Rate of syneresis of polysaccharides

EPS	Rate of syneresis (%)
L17	27.58
L20	45.15
L26	60.51
L27	62.12
xanthan gum	6.67
guar gum	7.51
carrageenan	15.01

Discussion

EPS produced by LAB is widely supplemented into fermented milk products to improve their rheological properties, mainly the texture, water holding properties, viscosity, and gelling properties. Yield of EPS produced depends on factors such as bacterial strain, medium (carbon and nitrogen sources) and growth conditions (Paulo et al. 2012). Vijayendra et al. (2008) reported that, at 30 °C, *Leuconostoc* sp. CFR 2181 produced EPS 22.5 $g\ L^{-1}$ in EPS medium was higher than in MRS medium (14 $g\ L^{-1}$) and more EPS (25.4 $g\ L^{-1}$) was produced when incubated at 22 °C. A homogenous, smooth body, higher viscosity and less

syneresis of fermented milk product was obtained when ropy LAB was employed in comparison to the use of non-ropy strains (Dierksen et al. 1997).

Conclusion

From the study, four EPS-producing LAB including strains L17, L20, L26 and L27 all of which were isolated from raw milk. Strain L17 and L20 demonstrated a ropiness colonies appearance while L26 and L27 are non-ropy strain. Strain L26, L20 and L17 gave good yields of EPS in the order of 19.38, 16.27 and 15.26 g L^{-1} respectively. Among these, strain L17 showed the best water-holding capacity. The preliminary resulted suggest that these EPS may have potential application in fermented milk.

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