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Characterization of highly branched dextran produced by *Leuconostoc citreum* B-2 from pineapple fermented product

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ABSTRACT

A strain *Leuconostoc citreum* B-2 was isolated from homemade fermentation product of pineapple and its polysaccharide yield was 28.3 g/L after cultivating the strain in Man-Rogosa-Sharpe (MRS) medium with 75 g/L sucrose. The exopolysaccharide (EPS) was characterized by gas chromatography (GC), Fourier-transform infrared (FT-IR) spectra, high-performance size-exclusion chromatography (HPSEC), nuclear magnetic resonance (NMR) spectroscopy and scanning electron microscope (SEM) analysis in present study. The monosaccharide composition of EPS was glucose and molecular weight was 3.77×10^6 Da. FT-IR and NMR spectra revealed that the B-2 EPS was composed of 75% α-(1 → 6) linked D-glucopyranose units existing in the main chain with 19% α-(1 → 3) branching and only a few α-(1 → 2) branching. The SEM of the dried EPS appeared irregular sheets with glittering surface and compact structure. Water solubility index and water holding capacity of B-2 EPS were 80% and 450%, respectively. All the mentioned characteristics suggested that the EPS has a potential application in the food, cosmetic and pharmaceuticals industry.

1. Introduction

In recent decades, microbial exopolysaccharide (EPS) has been attracted increasing attention of researchers because of their industrial, medical or food applications [1–3]. These EPSs may be found as a capsule attached to the bacteria or released to the environment as slime or both. Unattached EPS production is especially valuable for biotechnological industry as there is no need in costly procedures for removing from cell debris [4,5]. As lactic acid bacteria (LAB) has a generally regarded as safe (GARS) status [6], the EPS produced by LAB can be regarded as safe biopolymer and be divided into two groups: homopolysaccharides, contained only one type of monosaccharide, e.g. glucan (glucose) and fructan (fructose); and heteropolysaccharides, composed of repeating units that consist of two or more different monosaccharides [8,9]. Based on linkage bonds and nature of monomeric units, the homopolysaccharides can be clustered into four groups: α-D-glucans, β-D-glucans, fructans (e.g. levans, inulin) and others, like polygalactan [7]. Depending on the main chain glycosidic linkages, the α-D-glucans can be classified as (i) dextran, defined as homopolysaccharide of glucose that has α-(1 → 6) glycosidic linkage in the major chain with α-(1 → 2), α-(1 → 3) or α-(1 → 4) linked branches; (ii) mutan, which is characterized by consecutive α-(1 → 3) linkages in the linear backbone with α-(1 → 6) linked branch; (iii) alternan, which contains alternating α-(1 → 6) and α-(1 → 3) glucosidic linkages with some degree of α-(1 → 3) branching and (iv) reuteran which is a glucan mostly composed of α-(1 → 4) glucosidic bonds with some α-(1 → 6) branches [10,11]. It is known that the chemical characteristics of EPS might play a role in the beneficial properties, thus stimulated an interest towards the finding of new bioactive EPS.

At present, the glucan-producing strains are mainly *Streptococcus*, *Weissella*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Lactococcus* species. Among them, *Leuconostoc* species are the primary producers of the dextran that have a variety of structure and physicochemical properties and have a potential to be used as nutraceuticals. By giving examples, the characteristic of dextran synthesized by *Leu. citreum* S5 greatly depends on the sucrose concentration and the product can be utilized as the food ingredients with functional and rheological properties [12]; the dextran with highly α-(1 → 2) branching is usually used as prebiotic [13]; the well-known dextran produced by *Leu. mensenteroides* B-512F is widely used as blood-plasma substitute due to the low antigenicity, high water solubility and high biological stability in the human bloodstream [14]. In addition, other dextrans are applied in biological field, i.e. a series of classical dextrans as standards for size-exclusion chromatography and cross-linked dextrans used in the production of Sephadex columns [15,16]. Specially, dextran with alternating α-(1 → 6) and α-(1 → 3) linkages in the main chain which may be an ideal substitute for gum arabic to be used in the preparation of confectionaries, cosmetic, beverages, emulgator, and pharmaceuticals. Since

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structure and functions are intimately related, structural identification of the polysaccharides is contributed to studying its structure-activity relationship [17].

During the past decades, various EPS-producing *Lactobacillus* species especially *Leuconostoc mesenteroides* have been isolated from traditional sauerkraut and milk. Besides, the existing researches about glucan were mainly focused on exploring the glucans with high viscosity or exploring the linear dextrans with low molecular weight and good solubility. Thus far, only a few studies investigated branched dextrans with α-(1 → 3) and α-(1 → 2) linkages simultaneously. In order to enrich the source of EPS-producing strains and comprehensively study the structural characteristics of EPSs, the present work was aim to isolate and identify a dextran produced by *Leu. citreum* strain from pineapple fermented product, and to investigate the structural characteristics of EPS via Fourier-transform infrared (FT-IR) spectra, high-performance size-exclusion chromatography (HPSEC), nuclear magnetic resonance (NMR) spectroscopy. In addition, the physico-chemical properties of the EPS were elucidate, which will help to be applied in various food and pharmaceutical industries.

2. Material and methods

2.1. Materials

Man-Rogosa-Sharpe (MRS) medium, comprised of 20 g glucose, 10 g tryptone, 10 g beef extract, 5 g yeast extract, 5 g anhydrous sodium acetate, 2 g K_2HPO_4, 2 g ammonium citrate, 0.58 g $MgSO_4 \cdot 7H_2O$, 0.25 g $MnSO_4 \cdot H_2O$, 1 mL Tween 80 per litre (pH 6.9) was used for propagation [13]. The MRS medium containing 75 g/L sucrose (named MRS-S medium) was used for EPS production.

2.2. Screening EPS-producing strain and identification

The strain B-2 used in this study was obtained from homemade fermentation product of pineapple. Strain was routinely propagated in MRS medium at 30 °C for 20 h and used to produce EPS in MRS-S medium at 30 °C for 48 h. The strain B-2 was identified through morphological, biochemical tests and 16S rDNA sequence analysis. Total cellular DNA was extracted from freshly cultured bacteria and the 16S rDNA sequence was amplified by PCR method using universal primers 8F: 5′-AGAGTTTGATCATGGCTCAG-3′ and 1492R: 5′-ACGGTTACCTTGTTACGACTT-3′ [13]. The PCR reaction system containing 50 μL 2 × Taq Mix, 2 μL DNA, 44 μL double distilled water, 1 μL of each primer (10 pM) was conducted in a PCR thermal cycler (MyCycler; Bio-Rad Laboratories Inc., USA). The amplification was initiated at 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min. The final extension step was performed at 72 °C for 5 min. Finally, the termination reaction was cooled to 4 °C. The sequencing results of the PCR products were compared with the 16S rDNA gene sequence in Genbank of NCBI using BLAST.

2.3. Extraction and purification of the EPS

The B-2 strain was cultured into 500 mL MRS-S medium and then grown at 30 °C for 48 h with shake cultivation (80 r/min), which was inoculated at the amount of 1.5% (v/v). The methods of separation and purification were in consistent with method reported previously [18]. Briefly, the strain cells were removed by centrifuging at 4 °C and 4000 ×*g* for 60 min and then the supernatant was treated with three volumes of 95% cold ethanol and kept at 4 °C for 8 h. After centrifuging (4 °C, 12,000 ×*g*, 50 min), the precipitates were collected and dissolved in water (200 mL). Equivalent volume (200 mL) 10% (w/w) of trichloroacetic acid (TCA) was added and incessantly stirred for 4 h, followed by centrifuging at 4 °C and 12,000 ×*g* for 50 min to remove protein. The EPS was recovered by three volumes (1200 mL) of 95% (w/w) cold ethanol and then the pellet was gained through centrifuge and resuspended in deionized water. The crude EPS was dialyzed (MW cut-off 14,000 Da) to remove the impurity at 4 °C for 48 h. The retentate was further purified using gel-filtration chromatography (eluted with deionized water, flow rate 0.2 mL/min, detection wavelength was 220 nm) with a 1.6 cm × 50 cm Sephadex G-100 column. Finally, the purified EPS was lyophilized and yielded.

2.4. Monosaccharide composition analysis

Sugar composition of the EPS was analyzed by gas chromatography (GC). Briefly, 10 mg of purified EPS and 2 mL trifluoroacetic acid (TFA, 2 M) were sealed into ampoule bottle and hydrolyzed at 120 °C for 6 h. Then, the hydrolyzate was evaporated to remove the remanent TFA via rotary evaporation followed by lyophilization [19]. The dried hydrolyzate and four standard sugars (glucose, galactose, mannose, arabinose) were reduced with $NaBH_4$ and were acetylated using pyridine and acetic anhydride, then the derivatives were determined by Agilent 6820 GC with a flame ionization detector (FID) and OV-1701 capillary column (30 m × 0.32 mm × 0.5 μm). The operational conditions were described by Yang [13]. The monosaccharide of EPS was determined by comparing retention time of standard samples.

2.5. Determination of molecular weight

The molecular weight was detected by HPSEC with Shodex OH-park SB-805 column (8.0 mm × 300 mm, Japan) and refractive index detector (RID). T-series dextran standards (2 mg/mL) and the same concentration of sample were filtered through 0.22 μm filter membrane before injected. The column was used at 30 °C and the injection volume was 20 μL when the flow rate of pure water was 0.8 mL/min. The molecular mass was obtained according to standard curve which was performed by Shimadzu LC solution software.

2.6. FT-IR spectra analysis

FT-IR spectra analysis is a useful tool to ascertain functional group of polysaccharide. The EPS powder was mixed with dry potassium bromide (KBr) by a ratio of 1:100, then the mixture was pressed into pellet form. The spectrum was obtained using BIO-RAD FTS3000 IR spectra scanner with a distinguish ability of 1 cm^{-1} in the range of 400–4000 cm^{-1}.

2.7. NMR spectroscopy analysis

Further elucidation of polysaccharide structure was performed by NMR analysis. The spectra was recorded by a Bruker 400 liquid NMR spectrometer. According to the procedure described by Yang [13], the purified EPS (30–50 mg) was exchanged three times with deuterium oxide followed by dissolving in 0.55 mL D_2O and then placed in NMR tubes. All data were processed and analyzed using MestReNova software.

2.8. Scanning electron microscope (SEM) analysis

SEM was performed to observe the microstructure of EPS. The lyophilized EPS was fixed on specimen stage with double sided tape and then coated with a layer of Au (about 10 nm). The samples were viewed by scanning electron microscope (Hitachi, S-4800, Japan) with accelerating voltage of 15 kV.

2.9. Water solubility index (WSI)

WSI of the EPS was determined by the method of Kavitake et al. [20] with appropriate modification. The dried EPS sample of 200 mg was dissolved in 5 mL of Milli-Q water and stirred for 40 min in water bath at 40 °C to get a uniform suspension. Then the supernatant was