A NOVEL EXTRACELLULAR POLYSACCHARIDES FROM ACETIC ACID BACTERIUM, *Asaia bogorensis* NRIC 0311^{T}

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Abstract

Cell-surface polysaccharides are produced by a wide variety of bacteria. They can be attached to the cell membrane as the O-antigen of lipopolysaccharides (LPSs), from a capsule around the cell as capsular polysaccharides (CPSs), or be completely excreted as sexopolysaccharides (EPSs). Acetic acid bacteria are Gram-negative with rod shape and obligately aerobic bacteria. They belong to the family Acetobacteraceae as a branch of acidophilic bacteria in α-subdivision of Proteobacteria. Acetic acid bacteria have been known to produce a relatively large amounts of CPSs and EPSs, for example, *Gluconacetobacter xylinus* (former name *Acetobacter xylinum*) are able to produce thick pellicle consisting of bacterial cellulose. We have been reported that *A. aceti* IFO 3284 (reclassified as *A. pasteurianus* subsp. *lovaniensis*) produced novel CPS attached to the outer membrane and shown to be composed of two monosaccharides; glucose and rhamnose, at an approximately equimolar ratio and had molecular mass of higher than 700-kDa. Thermotolerant *A. tropicalis* SKU1100 produced a thick pellicle even at higher temperature such as 37^{o}C and 40^{o}C. The purified CPS was composed of glucose, rhamnose and galactose in an approximately equimolar ratio of 1:1:1 and had molecular mass of 120-kDa. *A. pasteurianus* produced CPS composed of glucose, rhamnose and xylose. EPS of *Kozakia baliensis* was purified which composed of two monosaccharides; glucose and galactose. The purified EPS was estimated to have an apparent molecular mass of higher than 700-kDa.

In this work, it was found that *Asaia bogorensis* NRIC 0311^{T} produced distinct polysaccharide from other acetic acid bacteria. The culture medium used for polysaccharide production comprised 5% glycerol as the carbon source. The polysaccharide was secreted into the culture medium and the character of the culture medium exhibited very low viscosity when compared with *K. baliensis* and then the EPS was harvested after incubation for 7 days. EPS from *A. bogorensis* NRIC 0311^{T} was precipitated from culture medium with alcohol and purified by two successive column chromatographies. The purified EPS was recovered more than 80%. Acid hydrolysis of purified EPSs with 2N trifuoroacetic acid at 121°C for 1 h shown to be mainly composed of two monosaccharides, glucose and mannose and trace of rhamnose. The purified EPS was estimated to have an apparent molecular mass of less than 100-kDa.

Keywords: extracellular polysaccharides, *Asaia bogorensis* NRIC 0311^T, acetic acid bacteria, polysaccharide purification

Introduction

Acetic acid bacteria are Gram-negative rod and obligately aerobic bacteria. They belong to the family Acetobacteraceae as a branch of acidophilic bacteria in α-subdivision of Proteobacteria. (De Ley et al., 1984) Members of the family Acetobacteraceae are *Acetobacter, Gluconobacter, Gluconacetobacter, Asaia,* and *Kozakia*. *Acetobacter* strains (including *Gluconacetobacter*) are well known as oxidative bacteria strongly oxidizing ethanol to acetic acid and thus have been used since historic ages for the vinegar fermentation. All *Acetobacter* species have a high ability of acetic acid production, which is dependent on the highly aerobic membrane-bound respiration. In static culture for vinegar production, a pellicle of acetic acid bacteria covers the surface of the fermentation culture. However, *Acetobacter* species have been known to mutate with high frequency and thus they can form two or more different types of colony after serial transfer. *A. xylinum* (reclassified as *Ga. xylinus*) is specie of the genus *Acetobacter*, which is well known to produce a pellicle consisting of bacterial cellulose and tightly attached to the cell surface. Pellicles produced by other *Acetobacter* species had also been believed to be composed of cellulose or cellulosic materials. However, it was discovered that *A. aceti* IFO 3284 R strain (reclassified as *A. pasteurianus* subsp. *lovaniensis*) (Moonmangmee et al., 2002a), *A. tropicalis* SKU1100 (Moonmangmee et al., 2002b), *A. pasteurianus* IFO 3191 produced a novel pellicle polysaccharide which is a cell-attached heteropolysaccharide (Moonmangmee et al., 2004) and also EPSs of *K. baliensis* was purified which composed of two monosaccharides, glucose and galactose. The purified EPSs was estimated to have an apparent molecular mass of higher than 700-kDa (Moonmangmee et al., 2008). *Asaia bogolensis* NRIC 0311^T, cells are Gram-negative rod, non-motile, strictly aerobic and catalase-positive acetic acid bacteria, which is isolated from tropical flowers by enrichment culture (Yamada et al., 2000). Although *A. bogorensis* NRIC 0311^T is a kind of acetic acid bacteria, it has unique properties as compared with other acetic acid bacterium such as *Acetobacter, Gluconobacter, Gluconacetobacter,* and *Kozakia*.

Methodology

Culture medium and cultivation conditions

Asaia bogorensis (NRIC 0311^T) culture was maintained on a potato-glycerol-$CaCO_3$, agar slant and plate that were prepared by adding 2% agar and 0.5% $CaCO_3$, to a potato medium consisting of 5 g of glucose, 10 g of yeast extract, 10 g of peptone, 20 g of glycerol and 100 ml of potato extract in 1-L of tap water (Matsushita and Ameyama, 1982). The cells maintained on the agar slant were inoculated into 5 ml of the potato liquid medium and cultivated with shaking for 24 h as a seed culture. Shaking culture was performed at 30°C with a rotary shaker set at 200 rpm. Pre-culture was performed by inoculating the seed culture into 100 ml of YPG medium (0.5% yeast extract, 0.5% peptone, and 2% glycerol) in a 500 ml Erlenmeyer flask and shaking for 24 h.

Exopolysaccharides production from *Asaia bogorensis* NRIC 0311^T

To prepare exopolysaccharides sample, the cultivation was performed in 5-L Erlenmeyer flask containing 1-L of YPG medium (5% glycerol, 0.5% peptone, and 0.5% yeast extract). Pre-culture was inoculated into the 1-L of YPG medium. It was then incubated in shaking culture for 7 days at 30°C.

Purification of *Asaia bogorensis* NRIC 0311^T EPS

The crude EPS of *A. bogorensis* NRIC 0311^T was isolated from culture supernatant fluids according to the modified procedure of Deeraksa et al. (2006) and Moonmangmee et al. (2008). The cells were removed by centrifugation (9,000 rpm for 15 min at 4°C). The culture supernatant fluids was concentrated to one third using ultra-filtration (20 kDa cutoff, Advantec), and collected by precipitation with 2 volumes of ethanol and then lyophilized. The lyophilized crude EPS sample was dissolved in small volume of 0.1 M NaCl. Then the fractionated was initially purified by DEAE–cellulose column chromatography and eluted with 25 mM Tris–HCl (pH 8.5). Polysaccharide containing fractions were determined for the presence of the total sugar by phenol-sulfuric acid method (Dubois et al., 1956). The fractions containing sugar were pooled, concentrated by ultra-filtration, and precipitated with 2 volumes of ethanol, respectively. The precipitated polysaccharide was then dissolved in small volume of 0.1 M NaCl and applied onto a Superdex S-200 column chromatography (ϕ1.6x70 cm). Elution was carried out at a flow rate of 60 ml h^{-1} with 0.1 M NaCl containing 0.1% SDS. The polysaccharide fractions were pooled and precipitated with 2 volumes of ethanol.

Measurement of molecular mass of the purified polysaccharide

The average molecular mass of the polysaccharide was estimated by size exclusion chromatography on a Superdex S-200 column chromatography (ϕ1.6x70 cm). Elution was carried out at a flow rate of 60 ml h^{-1} with 0.1 M NaCl containing 0.1% SDS, and the sugar content was determined colorimetrically at 490 nm by the phenol-sulfuric acid method (Dubois, et al., 1956). A calibration curve was obtained with pullulans standard (Showa Denko K.K., Tokyo): P20 (20 kDa), P50 (50 kDa), P100 (100 kDa), and P200 (186 kDa), respectively.

Acid hydrolysis

The monosaccharide composition was analyzed using purified polysaccharides (EPS and CPS). One milligram of each kind polysaccharide was hydrolyzed. The optimal conditions for hydrolysis of the purified EPS and CPS to release sugars composition were examined by TLC and the hydrolysates of a purified EPS and CPS fraction from *A. bogorensis* NRIC 0311^T were determined by hydrolysis with an aqueous solution of 2 N trifluoroacetic acid (TFA) for 1 h at 121°C in a glass vial that was tightly screw-capped (Teflon-sealed) (York et al. 1985). The resultant solution was evaporated to dryness at 40°C by means of a vacuum centrifuged evaporator. The dried product was dissolved in 1.0 ml of distilled water and evaporated to dryness again. The pellet thus obtained was dissolved in 0.1 ml of distilled water.

Determination of sugar composition by TLC

Samples were spotted (10-20 μg of starting material) on a silica gel plate (Silica gel 60, Merck Co., Germany) and developed by ascending chromatography using a mixture of n-propanol:water (85:15, v/v) solvent system. The spots were detected by spraying with freshly prepared aniline-diphenylamine-phosphoric acid reagent (4 ml of aniline, 4 g of diphenylamine, 200 ml of acetone, and 20 ml of 85% phosphoric acid) on the plates and baking for 5-10 min at 80-100°C (Dawson et al., 1986).

Chemical analyses

Total sugar content was determined by the phenol-sulfuric acid method (Dubois et al., 1956) using glucose as the standard. Total protein content was determined by the modified Lowry method (Dulley and Grieve, 1975) and also the amido black method (Schaffner and Weissmann, 1973) using a bovine serum albumin as the standard.

Results

Exopolysaccharides production from *Asaia bogorensis* NRIC 0311^T.

For exopolysaccharides production, *A. bogorensis* NRIC 0311^T can produce EPS after incubation for 7 days but the EPS produced from *A. bogorensis* NRIC 0311^T was distinct from EPS of *K. baliensis* when using glycerol as a carbon source. The polysaccharide was secreted into the culture medium and the character of the culture medium exhibited very low viscosity when compared with *K. baliensis*.

Purification of *Asaia bogorensis* NRIC 0311^T EPS.

Purification of the low viscosity crude extracellular polysaccharides produced by *A. bogorensis* NRIC 0311^T from the culture supernatant fluid of GYP was initially fractionated by an ion-exchange chromatography, DEAE-cellulose (Figure 1). The crude EPS was separated into two fractions, the main fraction (Peak I) in which passed through the column, and the miner fraction (peak II), which eluted from the column with approximately 0.20 M NaCl. Both fractions were non-viscous and contained high concentrations of neutral EPS in peak I and lower concentrations in peak II (Figure 1). The fractions containing sugar (peak I and peak II) were pooled, concentrated by ultra-filtration, and precipitated with 2 volumes of ethanol, respectively. The precipitated polysaccharide was then dissolved in small volume of 0.1 M NaCl and applied onto a Superdex S-200 column chromatography. Elution profile of purified EPS (peak I) showed a symmetric peak and EPS was eluted between fraction number 50-75 (Figure 2) and elution profile of peak II was the same to that of peak I (data not shown). The summary of isolation and purification procedures of EPS and CPS from *A. bogorensis* NRIC 0311^T is shown in Figure 3. EPS has been recovered with the yield of more than 80% (peak I) while the yield of CPS was around 20% (Figure 4).

Determination of sugar composition by Thin Layer Chromatography.

In order to examine the sugar composition of EPS and CPS, the 10-20 μg of purified of EPS and CPS were hydrolyzed by 2N trifluoroacetic acid at 121^{o}C for 1 h. Sugar components were identified by thin layer chromatography using sugar standards for identification. TLC was

developed with a n-propanol:water (85:15, v/v) as solvent system. Sugars were visualized by spraying with freshly prepared aniline-diphenylamine-phosphoric acid reagent on the plates, and baking for 5-10 min at 80-100°C (Dawson et al., 1986). The results showed that the EPS was composed of three different monosaccharides, main component are two monosaccharides; glucose and mannose and trace of rhamnose and CPS was composed of only glucose (Figure 5).

Characterization of the purified EPS.

Average molecular mass was estimated by size exclusion chromatography on a Superdex S-200 column chromatography (ϕ1.6 x 70 cm) using 0.1 M NaCl as eluent (fraction were collected at 60 ml h^{-1} and the sugar content was determine colorimetrically at 490 nm by phenol-sulfuric acid method. A calibration curve was obtained (data not shown) with the following standard pullulans (Showa Denko K.K., Tokyo, Japan): P20 (20 KDa), P50 (50 KDa), P100 (100 KDa), and P200 (186 KDa) respectively. The result showed that the average molecular mass of the purified EPS eluted as a single symmetric peak was estimated to be lower than 100 kDa (data not shown) where as the molecular mass of the purified CPS was not determined.

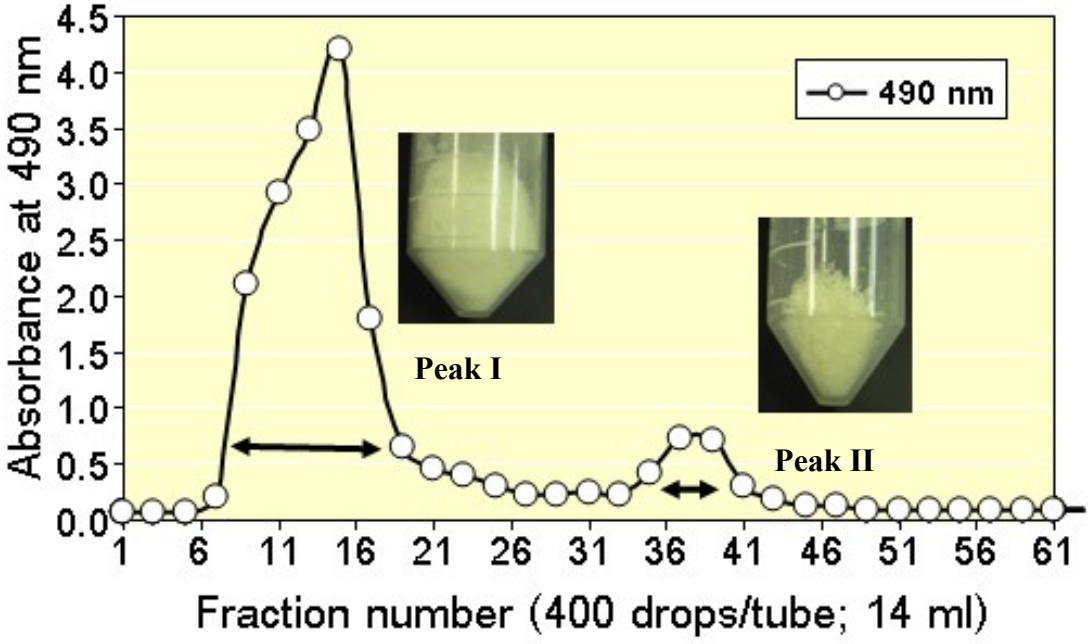

Figure 1 Ion-exchange chromatography of crude EPS of *Asaia bogorensis* NRIC 0311^{T} on DEAE-Cellulose. The crude EPS was obtained from the culture supernatant fluid of 7 days culture in GYP medium supplemented with 5% glycerol after ethanol precipitation. Conditions for elution are described in the text. Symbol: (o) optical density (absorbance at 490 nm) The partial purified EPS of fraction I (Peak I) and fraction II (Peak II; data not shown) were purified on Superdex S-200 column chromatography as described in Methodology and gel filtration was useful to remove protein contaminants.

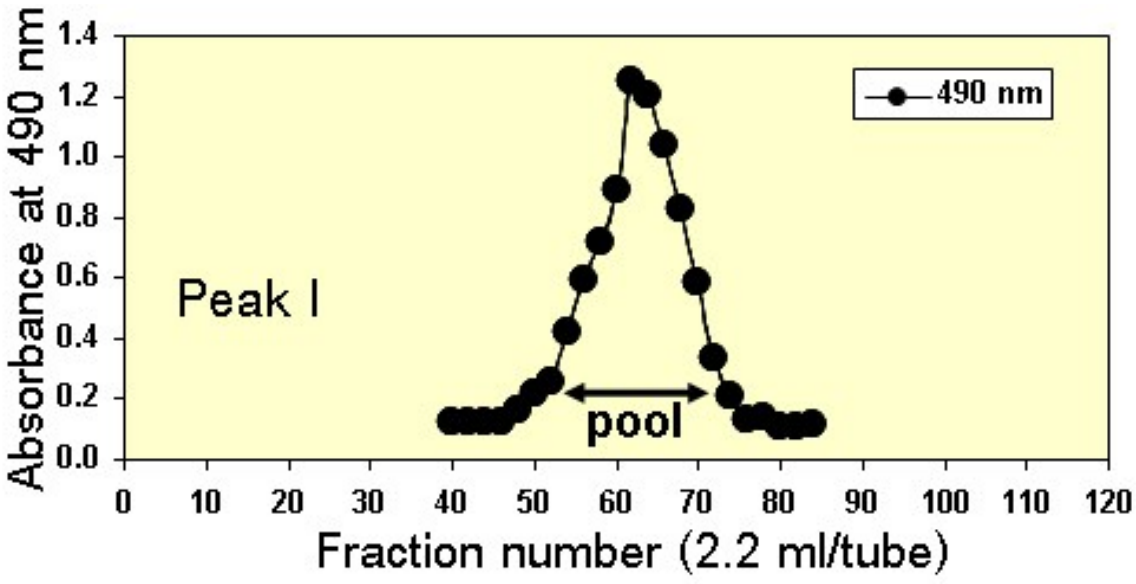

Figure 2 Gel filtration chromatography of partial purified EPS fraction I (peak I) on Superdex S-200 obtained after DEAE-Cellulose column chromatography. The conditions for chromatography were described in the text. Symbol: (●) optical density (absorbance at 490 nm)

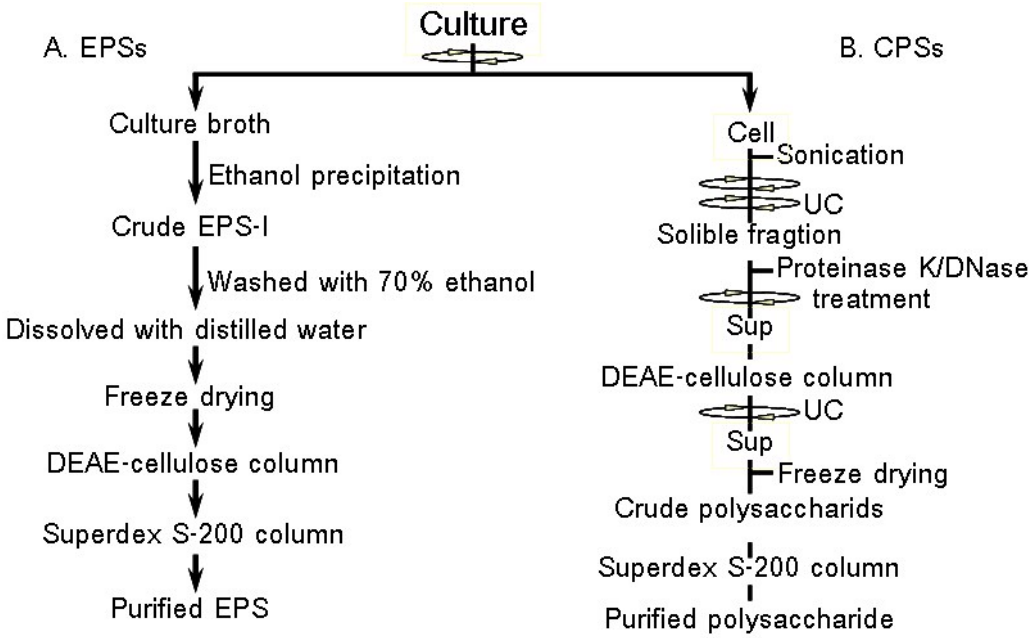

Figure 3 Summary of isolation and purification procedures of A) EPS and B) CPS from *Asaia bogorensis* NRIC 0311^{T}.

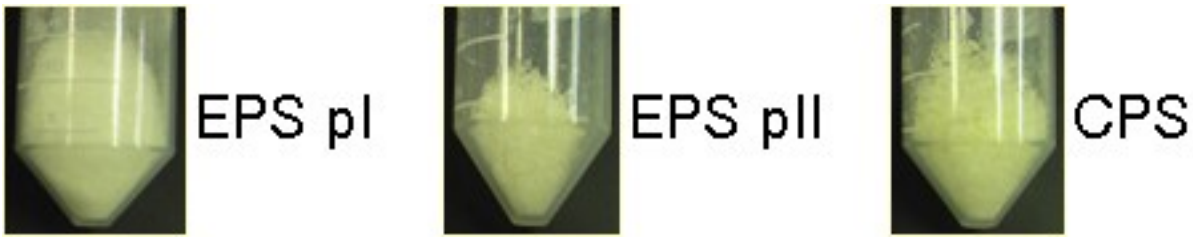

Figure 4 Purified EPS and CPS from *Asaia bogorensis* NRIC 0311^{T}.

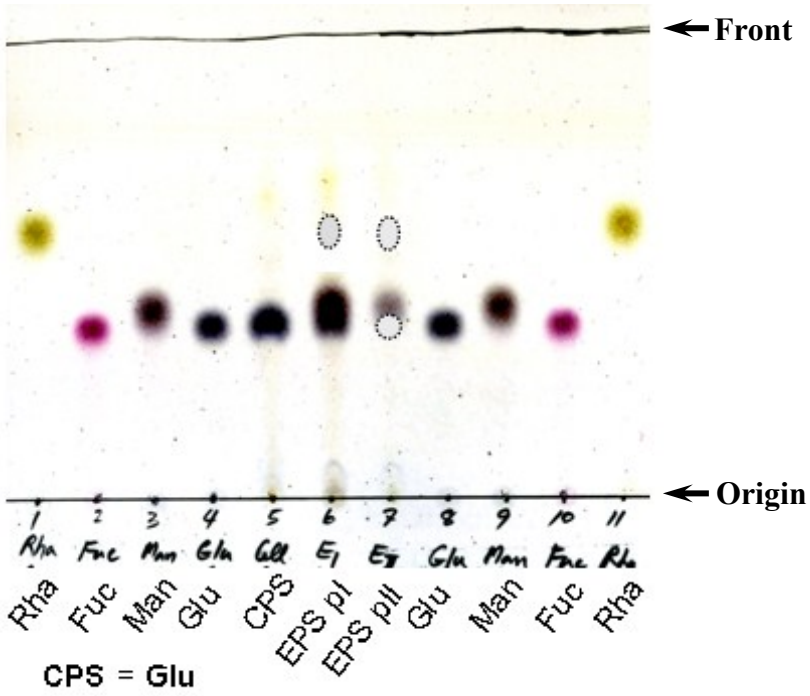

Figure 5 Thin layer chromatogram of acid-hydrolysis products of purified EPS and CPS from *Asaia bogorensis* NRIC 0311^{T}. Each sample was hydrolyzed with 2N TFA for 1 h at 121°C and developed on a silica gel 60/aluminium plate (Merck) with the solvent system: n-propyl alcohol/distilled water (85:15, v/v). Sugar spots were detected with freshly prepared aniline-diphenylamine phosphoric acid reagent (4 ml of aniline, 4 g of diphenylamine, 200 ml of acetone, and 20 ml of 85% phosphoric acid) on the plates, and baking for 5-10 min at 80-100°C (Dawson, et al., 1986). Lanes 1 and 11, authentic rhamnose; lane 2 and 10, authentic fructose; lane 3 and 9, authentic mannose; lane 4 and 8, authentic glucose; lane 5, the purified CPS and lane 6 and 7, the purified EPS.

Discussion and conclusion

Asaia bogorensis NRIC 0311^T produced both EPS and CPS, the EPS produced from *A. bogorensis* NRIC 0311^T was distinct from EPS of *Kozakia baliensis* when using glycerol as a carbon source. The polysaccharide was secreted into the culture medium and the character of the culture medium exhibited very low viscosity when compared to that of *K. baliensis* and then the EPS was harvested after incubation for 7 days (Moonmangmee et al., 2008). EPS from *A. bogorensis* NRIC 0311^T was precipitated from culture medium with alcohol and purified by two successive column chromatographies. The purified EPS was recovered more than 80% and CPS more than 20%. The detection reagent used for sugar determination gave specific spot colored in which fructose, rhamnose, mannose, glucose gave pink, yellow-green, blue-grey, and dark blue colour, respectively. The results clearly showed that monosaccharide contained in the purified EPS from *A. bogorensis* NRIC 0311^T gave dark blue, blue-grey and pale yellow-green spots. The sugar composition of the EPS was composed of three different monosaccharides, main component was glucose, mannose and composed trace of rhamnose and CPS was found only glucose spot. The purified EPS was estimated to had an apparent molecular mass of less than 100-kDa. The results obtained in this work were differed from Kato et al. (2007) which has been reported that cultivation of *A. bogorensis* NRIC 0311^T in sucrose medium, the water-soluble polysaccharide or fructan was produced. The composition of water-soluble polysaccharide seemed to be a homopolysaccharide contained only fructose. Thus, it was suggested that changing in the carbon source for growth, the polysaccharide composition of *A. bogorensis* NRIC 0311^T might be altered. The polysaccharide produced by *A. bogorensis* NRIC 0311^T using glycerol as the substrate had unique characteristics which distinct from other polysaccharide produced from *A. aceti* IFO 3284 (Moonmangmee et al., 2002a), *A. tropicalis* SKU1100 (Moonmangmee et al., 2002b), *A. pasteurianus* IFO 3191 (Moonmangmee et al., 2004), *K. baliensis* (Moonmangmee et al., 2008), and *Ga. xylinus* (Moonmangmee et al., 2002a).

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