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Original research article

Sucrase Activity and Exopolysaccharide Partial Characterization From Three *Weissella confusa* Strains

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ABSTRACT

Exopolysaccharides (EPSs) produced by lactic acid bacteria have been well known for their important economic value in food, pharmaceutical and health industries. Large extracellular enzyme sucrases are used by lactic acid bacteria to polymerize EPS, i.e. fructansucrase and glucansucrase. This study aimed to characterize sucrase activity of three *Weissella confusa* strains MBF8-1, MBF8-2 and MBFCNC-2(1), which were isolated previously from local beverages and their EPS products as well. All strains showed ability to form mucoid and slimy colonies by visual inspection on agar plate using raffinose as substrate suggesting that they possessed fructansucrase activity besides glucansucrase. Obtained EPS products were characterized by HPLC analysis after hydrolysis using 3% TCA at 100 °C for 1 hour, and by viscosity as well. All strains exhibited similar peak patterns, assuming that all of them possessed fructan EPS product. Supernatant and cell pellet were also analyzed by *in situ* activity assay performing periodic acid Schiff staining after polyacrylamide gel electrophoresis; only cell pellet showed sucrase activity. Viscosity observation showed that EPS products from all strains were able to increase the viscosity slightly.

1. Introduction

Exopolysaccharides (EPSs) are versatile metabolic products of lactic acid bacteria (LAB). Due to the wide diversity in composition and variation in their high molecular weights (10–1000 kDa), EPSs have found a myriad of multifarious applications in many industrial and medical sectors, including food and pharmaceutics (Nwodo *et al.* 2012; van Hijum *et al.* 2006; Velazquez-Hernandez *et al.* 2009).

EPSs produced by LAB are homopolysaccharide (HoPS) and heteropolysaccharide. HoPSs are composed of repeating units of a single monosaccharide, mainly glucose or fructose; the product is designated as glucan EPS and fructan EPS. Heteropolysaccharides are composed of repeating units consisting of two or more monosaccharides, mainly galactose, glucose and fructose (van Hijum *et al.* 2006). Glucan EPS and fructan EPS have been widely known, i.e. dextran, inulin, and levan. Enzyme involved in this HoPS synthesis is a large extracellular enzyme known as sucrase.

The EPSs produced by LAB exert various functions including adherence of cells to surfaces during colonization, protection from attack by antimicrobial agents, and as the communal life of biofilms (Nwodo *et al.* 2012; van Hijum *et al.* 2006). They involve the microbial quorum sensing control through regulation of gene expression for proteins involved in EPS biosynthesis (Vu *et al.* 2009). Jellification of cane sugar syrups by microbial origin had been reported 150 years ago (Pasteur 1861). This effect was caused by an EPS named dextran due to its positive rotary power. As one of bacterial extracellular biopolymer, EPS exists as the most abundant biopolymers classified based on their location relative to the cell, i.e. loosely attached and unattached extracellular polymeric substances corresponding with the cell in structural and functional relationship. The loosely attached EPSs serve structural and protective purposes for the bacteria, and in addition, may take the form of a covalently bound cohesive layer. On the other hand, they may be unattached completely excreted into the environment as slime (Nwodo *et al.* 2012).

Gene coding for glucansucrase (GS; *gtf*) and fructansucrase (FS; *ftf*) has been identified from three *Weissella confusa* strains originally isolated from Indonesian beverages, i.e. MBF8-1, MBF8-2 and

Abbreviations: EPS, exopolysaccharide; *ftf*, fructosyltransferase; FS, fructansucrase; *gtf*, glucosyltransferase; GS, glucansucrase; LAB, lactic acid bacteria; PAS, periodic acid Schiff.

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MBFCNC-2(1) (Malik *et al.* 2009; Malik 2012). The strain MBF8-1 carried two *gtf*, whereas strain MBF8-2 exhibited one *gtf* and one *ftf*. Unique features of these genes were discussed in previous study, and thus generated a challenge for further characterization of the FTF/GTF proteins. The strain MBFCNC-2(1) interestingly possessed *ftf* which showed highest similarity to a putative inulosucrase of *Lactobacillus reuteri*. LAB strains carrying multiple GS genes (*gtf*) in their genome sequence have been reported (Van Hijum *et al.* 2006). Moreover, FS inulosucrases are exclusively present in LAB, unlike other FS enzymes, levansucrases, which are widely distributed in both gram-positive and gram-negative bacteria. Fructan EPSs are of high demand for industrial sectors, thus making the enzyme involved in high interest to be explored from various sources and to be bio-engineered as well.

In this study, we reported the characterization of those strains regarding the production of EPS by sucrase activity, as well as simple preliminary GS/FS identification. This study exhibits steps to narrow down the FS/GS identification by their enzyme activities performing *in situ* activity assay after visual inspection, as well as characterization of the EPS product by using HPLC. Cross-check of this report with previous studies reveals the possibility for more FS and/or GS genes to be identified and studied.

2. Materials and methods

2.1. Bacterial strains and growth condition

All *W. confusa* strains, i.e. MBF8-1, MBF8-2 and MBFCNC-2(1) (Malik *et al.* 2009; Malik 2012) were cultivated at 32 °C in MRS (De Man *et al.* 1960) medium (Difco, Franklin Lakes, NJ), or in MRS-s medium (i.e. MRS medium with 100 g/L sucrose instead of 20 g/L glucose) for EPS production. For visual inspection, MRS medium modified with 5% raffinose (MRS-r), i.e. MRS medium with 50 g/L raffinose (Merck), and with 5% sucrose (MRS-s) was used specifically for FS enzyme activity detection (Malik *et al.* 2009; Malik 2012). Agar plates were made by adding 1.5% agar to the MRS medium.

2.2. Visual inspection assay for GS and FS activities

Visual inspection for FS enzyme activity was done as described previously (Malik *et al.* 2009).

2.3. EPS production and purification

The EPS production of three *W. confusa* strains were done by using MRS medium containing 10% sucrose as growth medium at 32 °C without agitation for 24 hours. Sucrose was separately autoclaved and then added to the sterilized MRS medium. Bacterial cell cultures were harvested by centrifugation at 4 °C and 5 000*g* for 30 minutes, the supernatants were collected and were kept at 4 °C for 2–3 hours until chilled. Two volumes of 96% ethanol were added to the supernatants and were incubated back at 4 °C for 8 hours. Cloudy appearance of coagulated EPS was collected by centrifugation at high speed (minimum 10,000 *g*) for 1 hour; the EPS precipitant was decanted from the clear liquid part, and re-dissolved in pure water by warming it up at 37 °C on a water bath. After cooling, this suspension was repeated for one more precipitation process in an attempt to purify the EPS. EPS precipitation process was repeated until no precipitate formed. All EPS precipitant suspensions obtained were pooled, and were then lyophilized and kept as dried purified EPS.

2.4. Identification of sugar composition

Composition of EPS sugar was analyzed by TLC and HPLC. For TLC analysis, silica gel GF350 plate was used as stationary phase, and a mixture of organic solvents were employed, i.e. n-butanol, ethanol, water (5:3:2). For HPLC analysis, a Prominence-20AB (Shimadzu, Japan) equipped with RI detector and an ion exchange CA-bonded SCR 101-C column have been employed with water only as moving phase, with resin polystyrene divynilbenzene as support. Hydrolysis of EPS was done by 3% TCA at 100 °C for 1 hour, and then was treated by deionization using cationic and anionic resins to remove all ion contaminants. HPLC was performed at 80°C and 1 mL/min with water as liquid phase. Reference samples used were glucose and fructose suspensions.

2.5. Measurement of viscosity

Observation of physical property (i.e. rheology/viscosity) was conducted using the LAB extracts harvested from the same fermentation process mentioned above, employing Ostwald viscometer. All *W. confusa* cultures in MRS-sucrose 10%, after incubation at 32 °C without agitation for 24 hours, were subjected to OD_{600} measurement in an attempt to adjust all cell cultures at the same concentration. Cultures were centrifuged at 4 °C and 5 000*g* for 30 minutes, and the supernatant was decanted. Medium MRS-sucrose 10% was used as reference, and the density of the reference as well as all sample supernatants were measured by a Piknometer before use in viscosity measurement.

2.6. *In situ* sucrase activity assay by SDS PAGE and periodic acid Schiff (PAS) staining

Supernatant and cell pellet obtained from liquid cultures were analyzed by performing polyacrylamide gel electrophoresis. The molecular mass was determined using the Laemmli system (Laemmli 1970) on 10% acrylamide (BioRad, USA) gel using electrophoresis AE-6530 mPAGE (Atto, Japan). Proteins were stained with Coomassie blue R-250 (BioRad), and molecular weight was estimated using protein marker PageRuler^R Prestained Protein Ladder^R (Fermentas, USA). Gel was run in duplicate, one for CBB staining and the other one for PAS staining. Raffinose buffer was used as substrate when the gel was incubated overnight, followed by staining with PAS reagent (Kralj *et al.* 2003). PAS staining consisted of incubation steps, first with 12.5% TCA for 30 minutes with slow and gentle agitation, followed by incubation with 1% periodic acid in 3% acetic acid for 50 minutes, and then with Schiff's Fuchsin-sulfite for 50 minutes, and last with 0.5% sodium metabisulfite for 30 minutes. Positive result can be observed as intensive pink to red band on the gel.

3. Results

3.1. GS and FS activities

The simple screening method for FS activity was done by visual inspection on agar plate using raffinose as substrate. Three *W. confusa* strains MBF8-1, MBF8-2 and MBFCNC-2(1), which were reported previously to possess mainly as FS gene carrier LAB as well as fructan EPS producers (Malik *et al.* 2009; Malik 2012), were able to produce a mucoid colony on plate as an indication of EPS product (Figure 1). The sucrase activity on MRS-sucrose 10% agar showed abundance of EPS product as seen on plate as mucoid and ropy colonies, whereas on MRS-raffinose 5% or MRS-raffinose 5% + glucose 5% showed lesser amount.

3.2. Isolation of EPS product

For the EPS production, the MRS-sucrose 10% liquid medium was chosen. Consecutive repeated simple purification steps were performed in this study to collect a rather pure EPS precipitate; no advanced technique needed for EPS product isolation. The dried EPS product obtained from isolation, purification and lyophilization appears as yellowish-white powdery precipitate as presented in Figure 2.

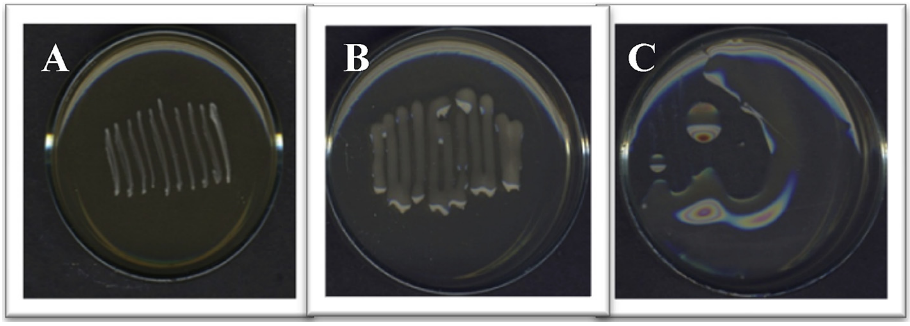

Figure 1. Visual inspection profile. Example is strain MBFCNC-2(1). (A) Growth on MRS agar. (B) Growth on MRS-raffinose 5%, mucoid appearance. (C) Growth on MRS-sucrose 10%, mucoid and slimy appearance.

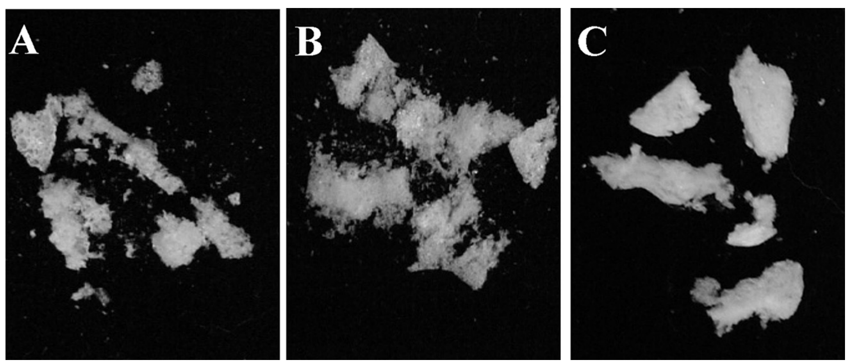

Figure 2. EPS product after isolation, purification and lyophilization appeared as yellowish-white powdery precipitate. (A) EPS from MBF8-1. (B) EPS from MBF8-2. (C) EPS from MBFCNC-2(1). EPS = exopolysaccharide.

3.3. Sugar composition of EPS product

Thin layer chromatography result showed interesting spots as shown in Figure 3. As references, dextran showed five spots, each of which has a Rf 0.4919; 0.3784; 0.2818; 0.2216; and 0.2000; whereas levan showed one spot at Rf 0.4919. The glucose has one spot at Rf 0.5189. Based on dextran to glucose Rf, we assumed that dextran cannot be hydrolyzed completely into the monomer, which is glucose, by the condition used. It can be concluded that the spots at Rf 0.3784; 0.2818; 0.2216; and 0.2000 were still the oligomers.

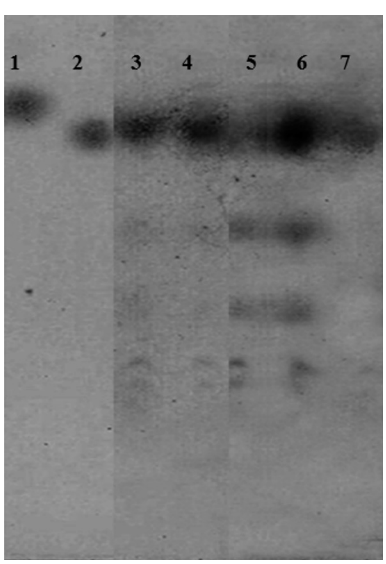

Figure 3. TLC results of EPS product hydrolyzed by HCl 1 N at 100°C for 30 minutes. Elution was done by using organic solvent mixtures, n-butanol-ethanol-water (5:3:2), whereas silica gel GF350 plate was used as solid phase. No. 1: glucose; 2: sucrose; 3: MBF8-1; 4: MBF8-2; 5: MBFCNC-2(1); 6: dextran; and 7: levan.

EPS samples from various isolates of LAB are also not completely hydrolyzed into sugar monomers and oligomers as shown in Figure 3. It was seen from Rf spots of EPS samples, i.e. 0.4919; 0.3784; 0.2818; 0.2216; and 0.2000 were not the same as Rf glucose, i.e. 0.5189.

All EPS products showed a strong peak at the retention time of fructose ($t_R = 8403$) confirming that all *Weissella* strains used in this study possess the ability to produce EPS fructan, as shown in Figure 4. The column used in this analysis was appropriate to analyze the carbohydrate, the polyalcohol, as well as mono-, di-, and oligosaccharides up to 11 degree of polymerization.

3.4. Sucrase activity of *W. confusa* strains by *in situ* gel assay

Analysis of the sucrase activity by *in situ* PAS staining was performed to show enzyme activity by its product EPS, as observed by an intensive pink band after PAS staining. Results of *in situ* activity assay of all cell pellet samples were presented in Figure 5.

3.5. EPS's role on viscosity

EPS containing supernatant of the fermentation culture was used with OD_{600} ranged from 1.3 to 1.5. After the biomass was separated by centrifugation, the supernatant containing the EPS was measured for its viscosity. The results of the measurements with Ostwald viscometer showed that the viscosity of the supernatant containing EPS from a number of LAB fermentation cultures was only slightly higher than water and 10% MRS-sucrose liquid solution as references, i.e. η of MBF8-1 = 1.029 cp; MBF8-2 = 1.063 cp; MBFCNC-2(1) = 1.015 cp, whereas η of references

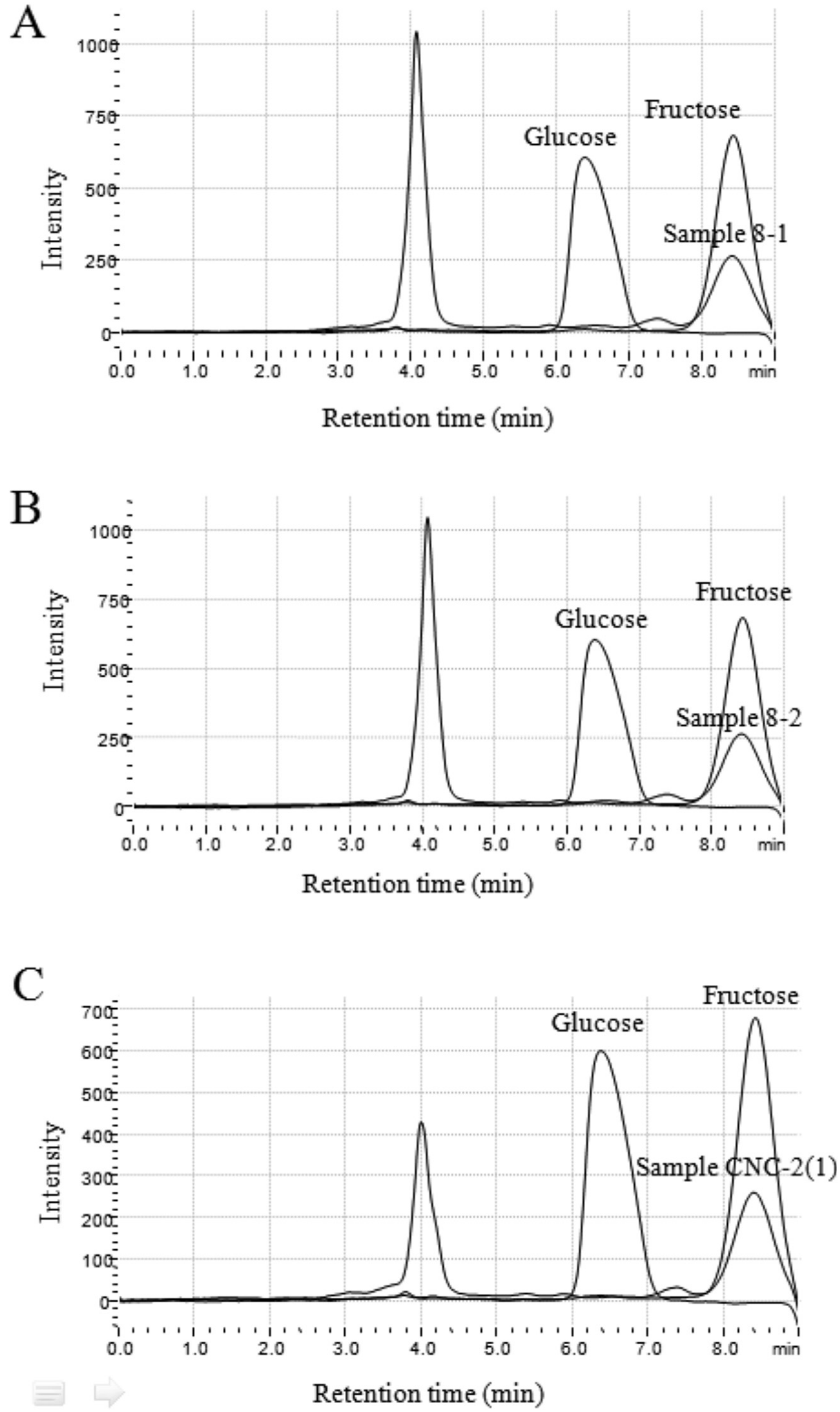

Figure 4. HPLC chromatogram of EPS analysis after hydrolysis with 3% TCA at 100°C for 1 hour. (A) EPS obtained from *Weissella confusa* MBF8-1. (B) EPS obtained from *W. confusa* MBF8-2. (C) EPS obtained from *W. confusa* MBFCNC-2(1). All EPS samples showed a peak at fructose retention time. Ca^{2+} cation exchange column was used with water as liquid phase at a rate of 1 mL/min. EPS = exopolysaccharide.

are distilled water = 0.836 cp and MRS-sucrose (10%) medium = 0.862 cp.

4. Discussion

The trisaccharide D-raffinose (αGal(1-6)αGlc(1-2)βFru) serves as fructosyl donor specifically for FS. The products resulting from FS-catalyzed hydrolysis of raffinose are melibiose (Gal-Glc) and free fructose (Meng & Fütterer 2008). Therefore FS must exist to build the fructan EPS appearing as slimy and mucoid colonies, or otherwise no EPS will be produced. However, there is an exception for FS from *L. gasseri* strains which were unable to ferment raffinose, whereas their respective recombinant enzymes used raffinose as a substrate as reported (Anwar *et al.* 2010).

In our previous studies, we reported that strain MBF8-1 harbors two *gtf* genes while MBF8-2 harbors one *gtf* and one *ftf* by molecular screening and identification (Malik *et al.* 2009; Malik 2012), as summarized in Table 1. In this study, however, it was determined by

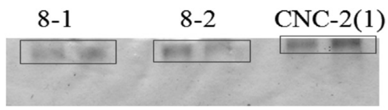

Figure 5. *In situ* activity performing PAS staining of polyacrylamide gel using sucrose as substrate appeared as violet−pink bands. 8-1: cell pellet of MBF8-1 culture; 8-2: cell pellet of MBF8-2 culture; CNC-2(1): cell pellet of MBFCNC-2(1) culture. PAS = periodic acid Schiff.

Table 1. Sucrase activity of *Weissella confusa* strains and EPS product

Strains			Molecular screening and identification		Visual inspection
gtf			HPLC analysis	Reference	*ftf*
MBF8-1	2	0	+	Fructose	Malik *et al.* 2009
MBF8-2	1	1	++	Fructose	Malik *et al.* 2009
MBFCNC-2(1)	1	1	+++	Fructose	Malik 2012

EPS = exopolysaccharide; *gtf* = gene for glucansucrase; *ftf* = gene for fructansucrase; + = positive to produce mucoid/slimy colonies.

HPLC analysis of the EPS product that MBF8-1 possibly carries an *ftf* gene. The molecular screening using the *ftf* degenerate primer was apparently unable to identify the *ftf* gene in MBF8-1. Two other strains determined in this study have been confirmed to carry *ftf* as reported by the previous molecular screening study (Malik *et al.* 2009; Malik 2012).

When the monomers compositions were analyzed by TLC, it was shown that some spot did not correspond to the reference monomer we used, i.e. glucose. There is a possibility of monomeric sugars actually been formed on hydrolysis conditions performed, but the amount of EPS product applied might be very low compared to the amount of glucose, so the monomer released cannot be detected with the reagent. To determine the monomer composition of the EPS, the HPLC method which was more sensitive is needed. Unfortunately, the glucan EPS could not be identified using TCA hydrolysis. TCA hydrolysis is a weak hydrolysis reaction, therefore, it could identify only fructose monomer released. Although we conducted several hydrolytic reactions using even stronger acid, e.g. HCl, we did not succeed in producing good and reproducible HPLC data for glucose monomer. TCA hydrolysis is a weak hydrolysis reaction, therefore, it could successfully identify only the released of fructose monomer.

Periodic acid is an oxidizing agent that can break bonds in the carbon chains of different chemical structures and then convert them into dialdehydes. PAS reaction works with the principle that the periodic acid oxidizes carbon−carbon bonds of carbohydrates where the carbon atom carries a hydroxyl group or a primary or secondary amine to produce dialdehyde which can react with Schiff's reagent. Schiff's reagent will stain the oxidized areas pink to red. The pink to red color that occurs is the new compound resulting from the combination of fuchsin (from Schiff's reagent) with dialdehyde, not from the re-oxidation of fuchsin (Robbins *et al.* 1980). The band showed in the polyacrylamide gel was obviously confirmed as the EPS product of the fructansucrase activity on substrate raffinose.

In this study, viscosity measurement was carried out by employing Ostwald viscometer because EPS containing supernatant is diluted liquid and has Newton fluid properties. Newton fluid is a fluid with constant viscosity, wherein the shear rate of the sample is directly proportional to the shear stress (Sinko & Singh 2011). The supernatant's viscosity is influenced by various factors, including the amount of EPS produced, the molecular weight, the degree of polymerization of EPS produced by different strains, and the type of glycoside bonds of the EPS. However, study of EPS from *S. thermophilus* was reported as a non-Newtonian behavior (shear-thinning) of the EPS solution in contrary, as it showed drastic decrease in viscosity of the EPS solution with increasing shear rate (Zhang *et al.* 2011). Ostwald viscometer was used to measure the viscosity of the EPS sample, because EPS containing supernatant is diluted liquid and has Newton fluid properties. Nevertheless, the results of the viscosity measurements showed that the EPS produced by all strains can play a role as thickening agents, which can be developed for applications in various industries, such as pharmaceutical and cosmetics industries, as well as the food industry, and it has been widely reported (Ruas-Madiedo *et al.* 2002; Sutherland 2001)

The possible number of glucan and/or fructan in the various strains is still under question, along with the role of EPS synthesis in their individual hosts. In the context of EPS diversity related to the sugar branching, the sucrases involved could also vary. It was reported that three GS enzymes GTFA, GTF180 and GTFML1 are highly similar but they synthesize glucans with different glucosidic linkages (Kralj *et al.* 2004). Furthermore, the probiotic bacterium *Lactobacillus reuteri* 121 produces two fructosyltransferase enzymes, a levansucrase and an inulosucrase; although they share high sequence similarity, they differ significantly in the type and size distribution of fructooligosaccharide products synthesized from sucrose, and in their activity levels (Anwar *et al.* 2012).

LAB, specifically genus *Weissella*, has a great potential for future studies focusing on the bio-engineering of EPS products resulting in EPS products with single unique glycoside bond and producing more physical characters that have more benefits of the application, such as gel-forming-polymers (Moulis *et al.* 2006). Through the use of molecular techniques such as mutagenesis and cloning of genes coding sucrase enzymes, this goal can be achieved. FS enzyme involved in certain fructan EPS synthesis such as inulin is of high interest to be studied. Inulin and its derivatives have been well studied for decades, predominantly in health industries for nutraceutical application, which is well known as prebiotic. This EPS is also of high interest in pharmaceutical industry for protein/peptide (biotech) drug protectant. Hence, the progress for the development of tailor-made fructan EPS inulin and its derivatives (fructooligosaccharide) is urgent.

Acknowledgments

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Authors declare that they have no conflict of interest.

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