Decoding the Capability of *Lactobacillus plantarum* W1 Isolated from Soybean Whey in Producing an Exopolysaccharide

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**ABSTRACT:** This study aims at producing exopolysaccharides (EPS) from a lactic acid bacterium. The soybean whey-isolated *Lactobacillus plantarum* W1 (EPS-W1), which belongs to genus *Lactobacillus*, is identified using the phenylalanyl-tRNA sequencing method. Of all the examined strains, R-49778 (as numbered by BCCM/LMG Bacteria Collection, Ghent University, Belgium) showed the highest capability of producing exopolysaccharides. Structural characterization revealed a novel exopolysaccharide consisting of repeating units of \( \rightarrow 6 \)-d-Glcp-(1→3)-d-Manp-(1→3)-d-Manp-(1→2)-d-Glcp-(1→). This discovery opens up avenues for the production of EPS for food industries, functional foods, and biomedical applications.

1. INTRODUCTION

Lactic acid bacteria (LAB)-produced exopolysaccharide (EPS) compounds have emerged as a topic of interest in the food industry owing to the outstanding rheological and bioactive properties of the obtained products. According to Wang et al., EPS from *Lactobacillus plantarum* 70810 significantly inhibits the proliferation of HepG-2, BGC-823, and especially HT-29 tumor cells. Numerous efforts have been devoted to produce EPS from various species of the LAB e.g., *L. gasseri* FR4 and *L. fermentum* LL2. The achieved EPSs have multiple functions in food industries; they are used as antioxidants, vicosifying agents, antimicrobial agents, anticancer agents, and immunomodulatory agents. Considering the huge population comprising the LAB, there are several possibilities for foraging a novel EPS, which can be of significance in food and biomedical industries.

In 2000, González et al. isolated and identified 249 LAB strains from freshwater fish using traditional methods. Of these 249 strains, 237 were rods and 12 were cocci. Of the 237 rod strains, 226 belonged to *Carnobacterium*, and 11 belonged to *Lactobacillus*. The same method was also employed by Desiye and Abegaz (2013) to identify 107 lactic acid bacterial strains isolated from Tef Batter, a fermented product in Ethiopia. So far, utilization of *Lactobacillus plantarum* has been rarely reported. The EPS-producing capability of this novel strain is still unclear and requires intensive investigation.

In this study, we isolated *Lactobacillus plantarum* W1 (EPS-W1) from soybean whey and used it to produce EPS for the first time. We found that the *Lactobacillus plantarum* W1 (EPS-W1) strain R-49778 (as numbered in BCCM/LMG Bacteria Collection, Ghent University, Belgium) has an efficient way of producing EPS. More importantly, characterization of the obtained EPS disclosed a novel structure, which consists of repeating units of \( \rightarrow 6 \)-d-Glcp-(1→3)-d-Manp-(1→3)-d-Manp-(1→2)-d-Glcp-(1→). This finding opens a new avenue for the utilization of novel EPS in food industries.

2. RESULTS AND DISCUSSION

2.1. EPS Production Ability of Isolates. Figure 1 shows the exopolysaccharide synthesized by the LAB strains. A W1 strain, which shows the best EPS production corresponding to 97.44 mg/L, was selected for further experiments. The EPS-producing ability of the W1 strain is similar to that of *L. plantarum* KFS or YW11, with the obtained amounts of EPS in culture conditions being 97.44, 95.58, and 90 mg/L, respectively. The EPS-producing ability of the W1 strain was higher than that of *L. plantarum* WCFS1 and *L. plantarum* 70810 and lower than that of other strains such as *L. plantarum* FR4 and *L. fermentum* LL2. The achieved EPSs have multiple functions in food industries; they are used as antioxidants, vicosifying agents, antimicrobial agents, anticancer agents, and immunomodulatory agents.

Received: October 28, 2020
Accepted: December 7, 2020
Published: December 17, 2020
DM5, *L. plantarum* RJF4, and *L. plantarum* ZDY2013. Thus, the EPS-producing ability of different *L. plantarum* strains was different.

### 2.2. Identification of the W1 Strain by PheS

Strain W1 was identified with PheS sequencing. The sequence of a part of the PheS gene searched with BLAST identified W1 as *L. plantarum* with 100% similarity when compared with the NCBI accession number of reference species CP029349.1, CP028977.1, CP021086.1, CP028421.1, and CP028424.1. This strain had the strain number R-49778 in BCCM/LMG Bacteria Collection, Ghent University, Belgium.

### 2.3. Molecular Mass of EPS

The results of gel permeation chromatography indicate that EPS-W1 is a heterogeneous EPS with a polydispersity index value of 1.82, which was determined from the ratio of weight average molecular weight (Mw) to number average molecular weight (Mn). The average molecular weight of EPS from *L. plantarum* W1 was approximately 1.11 × 10^6 Da (Figure 2). EPS obtained from some other *L. plantarum* also showed similar molecular weights. The average molecular weights of EPS from *L. plantarum* YW11 and *L. plantarum* YW32 were 1.1 × 10^5 and 1.03 × 10^5 Da, respectively. Some EPSs have smaller sizes, such as 10^4–10^5 Da for those from *L. plantarum* BC-25 or *L. plantarum* Lp90. A few *L. plantarum* synthesized larger EPS. The average molecular weight of EPS from *L. plantarum* STK109 was 2.1 × 10^6 Da and from *L. plantarum* DM5 was 1.11 × 10^6 Da. Thus, EPS from *L. plantarum* W1 was of medium size according to published information.

### 2.4. Methylation Analysis and Monosaccharide Composition of EPS

Gas chromatography-coupled mass spectrometry (GC–MS) was employed to identify the composition of EPS via methylation (see the experimental section). Methylation analysis showed that EPS-W1 had five components: 1,5-diacetyl-2,3,4,6-tetra-O-methyl-glucitol, 1,5,6-triacetyl-2,3,4-tri-O-methyl-D-mannitol, 1,2,5,6-tetraacetyl-3,4-di-O-methyl-D-glucitol, 1,3,5-triacetyl-2,4,6-tri-O-methyl-D-glucitol, and 1,3,5-triacetyl-2,4,6-tri-O-methyl-mannitol were the main methylated sugar derivatives. Methylation analysis showed the presence of Glcp, 6-substituted manp, 2,6-substituted Glcp, 3-substituted Glcp, and 6-substituted manp suggesting a repeating linear hexasaccharide with all residues in the pyranose ring form. These results indicated that EPS-W1 mainly consisted of (1→6)-linked glucosyl, (1→6)-linked mannosyl, (1→2,6)-linked glucosyl, (1→3)-linked glucosyl, and (1→3)-linked mannosyl.

Monosaccharide composition analysis of the EPS showed the presence of glucose and mannose in EPS-W1. Most of the EPSs from *L. plantarum* are heteropolysaccharides. These heteropolysaccharides are usually composed of two or three monosaccharides. Some EPSs of the same species consist of many types of monosaccharides. For example, EPS from *L. plantarum* YW32 consists of four monosaccharides, and *L. plantarum* WCFS1 has five monosaccharides. According to the overview of Jiang and Yang (2018), the sugar compositions of EPS from *L. plantarum* were very varied. However, some of the *L. plantarum* strains can synthesize EPS to be a homopolysaccharide. For example, EPS produced by *L. plantarum* DM5 is a glucan, and EPS of *L. plantarum* 70810 is a galactan. The publications revealed that the monosaccharide composition of EPS-W1 can be considered to differ from that of EPS obtained from other *L. plantarum* strains. The variation in monosaccharide compositions of EPS from *L. plantarum* may be related to the activities of enzymes catalyzing for biosynthesis of EPS. In this study, activities of enzymes that catalyze to synthesize fructose may be much lower than those of enzymes catalyzing the mannose synthesis so that fructose residues do not appear in the EPS-W1 structure.

### 2.5. 1H NMR and 13C NMR Analysis of EPS-W1

1H NMR, 13C NMR, and 2D NMR (HMBC, HSQC) spectra of EPS-W1 were used to further determine the component and structure of the EPS. The anomeric region on 1H NMR of EPS-W1 showed six anomic proton signals (chemical shifts at 5.76, 5.67, 5.59, 5.56, 5.55, 5.37 ppm) and an oxymethyl group signal (3.7–4.5 ppm). This is an indication of the presence of six sugar components, supporting a hexasaccharide repeating unit (Figure 3). In the 13C NMR spectrum, EPS-W1 showed six anomic carbon signals at 103.4, 101.3, 94.7, and 94.4 ppm, where the peak at 101.3 was seen for the HSQC (Figure 5) correlation spectrum. The C2–C6 resonance signals were located in the region of 61.8–73.9 ppm (Figure 4). Meanwhile, the correlation peaks in HSQC demonstrated that the anomeric carbon peaks at 103.4, 102.7, 101.3, 99.1, 94.7, and 94.4 ppm were prevalent to the anomeric proton 5.41; 5.37; 5.60; 5.76; 5.67; and 5.56 ppm. The pyranose/furanose rings of monosaccharides were determined based on methylation analysis. The absolute configuration of monosaccharides can be determined based on the chemical shifts of anomeric protons (the values of the H1 proton). Thus, the values of the H1 proton, which are less than 5.0 ppm, could be assigned to α-pyranose in the absolute configuration of monosaccharides. In contrast, values that are higher than 5.0 ppm correspond to β-pyranose.
1H and 13C NMR chemical shifts (δ, ppm) of EPS-W1 (Table 1 and Figure 5) were determined by the 1H−13C HSQC NMR and 1H−13C HMBC spectrum (Figure 6). The 1H−13C HSQC NMR spectrum showed that cross-peaks were confirmed in the region for anomeric resonances δC 61.8−103.4 ppm and δH 4.13−5.76 ppm. Sugar residues A were indicated by the signals at δH 5.76/101.3, at δH 4.36/67.8, at δH 4.42/70.5, at δH 4.35/71.9, and at δH 4.13/61.8. Sugar residues B were indicated by the signals at δH 5.67/94.7, at δH 4.57/73.2, at δH 4.42/67.8, at δH 4.38/67.8, at δH 4.36/71.0, and at δH 4.37/73.9, and the presence of the signals at δH 5.55/

![Figure 3. 1H NMR spectrum of EPS-W1.](image3)

![Figure 4. 13C NMR spectrum of EPS-W1.](image4)

![Figure 5. 2D 1H−13C HSQC spectrum of EPS-W1.](image5)

### Table 1. 1H and 13C NMR Chemical Shifts (δ, ppm) of EPS-W1 Recorded in D2O at 353 K

| sugar residue          | H-1  | H-2  | H-3  | H-4  | H-5  | H-6  |
|------------------------|------|------|------|------|------|------|
| A α-α-D-glucopyranoside (1→) | 5.76 | 4.36 | 4.42 | 4.43 | 4.35 | 4.13 |
| B α-6)α-α-D-mannopyranoside (1→) | 5.67 | 4.57 | 4.42 | 4.38 | 4.36 | 4.32 |
| C α→2,6)α-α-D-glucopyranoside (1→) | 5.55 | 4.26 | 4.31 | 4.39 | 4.36 | 4.24 |
| D α→3)α-α-D-glucopyranoside (1→) | 5.37 | 4.20 | 4.19 | 4.39 | 4.42 | 4.42 |
| E α→6)α-α-D-mannopyranoside (1→) | 5.39 | 4.47 | 4.33 | 4.48 | 4.29 | 4.28 |
| F α→3)α-α-D-mannopyranoside (1→) | 5.56 | 4.52 | 4.32 | 4.43 | 4.31 | 4.32 |
| sugar residue          | C-1  | C-2  | C-3  | C-4  | C-5  | C-6  |
|------------------------|------|------|------|------|------|------|
| A α-α-D-glucopyranoside (1→) | 101.3| 67.8 | 70.5 | 71.1 | 71.9 | 61.8 |
| B α-6)α-α-D-mannopyranoside (1→) | 94.7 | 73.2 | 67.8 | 67.8 | 71.0 | 73.9 |
| C α→2,6)α-α-D-glucopyranoside (1→) | 103.4| 70.5 | 71.1 | 67.7 | 71.5 | 67.8 |
| D α→3)α-α-D-glucopyranoside (1→) | 94.4 | 71.5 | 72.8 | 63.4 | 71.9 | 71.4 |
| E α→6)α-α-D-mannopyranoside (1→) | 99.1 | 69.4 | 70.5 | 73.2 | 71.5 | 67.8 |
| F α→3)α-α-D-mannopyranoside (1→) | 102.7| 70.5 | 67.8 | 71.4 | 73.2 | 67.8 |
103.4, at δ 4.26/70.5, at δ 4.31/71.1, at δ 4.39/67.7, at δ 4.36/71.5, and at δ 4.24/67.8 were provided for sugar residues C. Similarly, sugar residues D were indicated by the signals at δ 5.37/94.4, at δ 4.20/71.5, at δ 4.19/72.8, at δ 4.39/63.4, at δ 4.42/71.9, and at δ 4.42/71.4. These signals δ 5.59/99.1, δ 4.74/69.4, δ 4.33/70.5, δ 4.48/73.2, δ 4.29/71.5, and δ 4.29/67.8 were provided for sugar residues E. Sugar residues F were indicated by the signals at δ 5.56/102.7, at δ 4.52/70.5, at δ 4.32/67.8, at δ 4.43/71.4, at δ 4.31/73.2, and at δ 4.32/67.8.

The positions of H 2−6 and C 2−6 of sugar residues were determined through the correlations in the HMBC NMR spectrum, as shown in Table 2. The substituted hydroxyl groups are in position 6 of sugar residues B, position 2,6 of sugar residues D, position 6 of sugar residues C, position 3 of sugar residues F, and position 3 of sugar residues A. These results are consistent with the methylation analysis.

The sugar chain of EPS-W1 was determined through the HMBC (Figure 6) and NOESY spectrum (Figure 7). The HMBC spectrum of EPS-W1 showed the correlation between an anomic proton A H-1 (δ 5.76) and carbon E C-6 (δ 67.8), proton anomer E H-1 (δ 5.59) and carbon C C-3 (δ 71.1), proton anomer C H-1 (δ 5.55) and carbon F C-3 (δ 67.8), proton anomer B H-1 (δ 5.67) and carbon C C-2 (δ 70.5), proton anomer F H-1 (δ 5.56) and carbon D C-3 (δ 72.8), and proton D H-1 (δ 5.37) and carbon A C-6 (δ 61.8). Additionally, the NOESY spectrum (Figure 7) showed strong correlations between E H-1 (δ 5.59) and C H-3 (δ 4.31), C H-1 (δ 5.55) and F H-3 (δ 4.32), B H-1 (δ 5.67) and C H-2 (δ 4.26), and F H-1 (δ 5.56) and D H-3 (δ 4.19).

The mentioned characterizations and analysis demonstrate a novel EPS, which is composed of a hexasaccharide repeating unit of →6)-D-GlcP-(1→; →3)-D-Manp-(1→; →3)-D-GlcP-(1→ and a branch of →6)-D-Manp-(1→; →2)-D-GlcP-(1→. This study identifies a novel strategy to fabricate EPS, which could have potential for applications in food industries.

4. MATERIALS AND METHODS

4.1. Microorganisms and Chemicals. Twenty different samples of soybean whey were collected aseptically from households throughout Hue city. The samples were kept at 4 °C before isolating bacteria from them. The bacterial strains were isolated and maintained in MRS broth (Oxoid, Milan, Italy).

DEAE-cellulose-52, a product of Thermo Fisher Dialysis membranes (Mw cutoff, 8000–14,000 Da), was purchased from Bum Han Commercial Co. All of the reagents, solvents, and chemicals used in this work were of analytical grade and were purchased from Sigma-Aldrich Co. (USA). These included Sephadex G-100 (CAS number: 9050-94-6), lactose (CAS number: 63-42-3), (CH3)2SO (CAS number: 67-68-5), (CH3)2SO4 (CAS number: 77-78-1), NaBH4 (CAS number: 16940-66-2), anhydride acetic (CAS number: 108-24-7), trifluoroacetic acid (CAS number: 76-05-1), and trichloroacetic acid (CAS number: 76-03-9). All other reagents were of analytical grade and were used without further purification.

4.2. Isolation of Lactic Acid Bacteria. In a typical experiment, 15 g of each sample was dispersed in Ringer’s solution (Sigma-Aldrich, Milan, Italy), serially diluted prior to plating onto MRS agar (Oxoid, Milan, Italy). Then, the as-prepared sample was incubated for 48 h in an incubator (model UN55, Memmert, Germany) at 37 °C. Afterward, 10–15 colonies, which were randomly selected, were plated on MRS agar to identify the pure culture by colony morphology. Gram staining and catalase reaction were employed to evaluate all isolates. Gram-positive and catalase-negative isolates are denoted as LAB and stored at −80 °C using Microbank vials (Pro-Lab Diagnostics, Richmond Hill, ON, Canada) for later characterizations.

4.3. Growth of the Microorganisms and Analysis of EPS Yields. EPS was obtained from LAB following the protocol of Wang et al. (2014)22 and Lynch et al. (2014)25 with some modifications. For determination of the EPS-producing ability, LAB was inoculated in MRS with an initial cell density of 10⁶ CFU/mL and incubated at 37 °C for 48 h (non-shaken, non-aerated) in an incubator (model UN55, ...
Table 2. Significant $^1$H/C Connectivities Observed in the HMBC Spectrum for Protons/Carbons of the Sugar Residues in EPS-W1

| sugar residue | sugar linkage | connectivities (H/C (ppm)) |
|---------------|---------------|---------------------------|
| A             | α-α-glucopyranoside (1→6) | H-2A (δ$_H$ 4.36) to C-3A (δ$_C$ 70.5); H-3A (δ$_H$ 4.42) to C-4A (δ$_C$ 71.1). C-2A (δ$_C$ 67.8); H-4A (δ$_H$ 4.43) to C-5A (δ$_C$ 71.9). C-3A (δ$_C$ 70.5); H-5A (δ$_H$ 4.35) to C-6A (δ$_C$ 61.8). C-4A (δ$_C$ 71.1). H-6A (δ$_H$ 4.13) to C-5A (δ$_C$ 71.9) |
| B             | →6)-α-α-mannopyranoside (1→6) | H-2B (δ$_H$ 4.57) to C-3B (δ$_C$ 67.8). C-1B (δ$_C$ 94.7); H-3B (δ$_H$ 4.42) to C-4A (δ$_C$ 71.1). C-2B (δ$_C$ 73.2); H-4B (δ$_H$ 4.38) to C-5B (δ$_C$ 71.0). C-3B (δ$_C$ 67.8); H-5B (δ$_H$ 4.36) to C-6B (δ$_C$ 73.9). C-4B (δ$_C$ 67.8); H-6B (δ$_H$ 4.37) to C-5B (δ$_C$ 71.0) |
| C             | →2,6)-α-α-glucopyranoside (1→6) | H-2C (δ$_H$ 4.26) to C-3C (δ$_C$ 71.1). C-1E (δ$_C$ 99.1); H-3C (δ$_H$ 4.31) to C-4C (δ$_C$ 67.7). C-2C (δ$_C$ 70.5); H-4C (δ$_H$ 4.39) to C-5C (δ$_C$ 71.5). C-3C (δ$_C$ 71.1); H-5C (δ$_H$ 4.36) to C-6C (δ$_C$ 67.8). C-4C (δ$_C$ 67.7); H-6C (δ$_H$ 4.24) to C-5C (δ$_C$ 71.5). C-1B (δ$_C$ 94.7) |
| D             | →3)-α-α-glucopyranoside (1→6) | H-2D (δ$_H$ 4.20) to C-3D (δ$_C$ 72.8); H-3D (δ$_H$ 4.19) to C-4D (δ$_C$ 63.4); C-2D (δ$_C$ 71.5). C-3C (δ$_C$ 71.1); H-4D (δ$_H$ 4.39) to C-5D (δ$_C$ 71.9). C-3D (δ$_C$ 72.8); H-5D (δ$_H$ 4.42) to C-6D (δ$_C$ 71.4). C-4D (δ$_C$ 63.4); H-6D (δ$_H$ 4.42) to C-5D (δ$_C$ 71.9). |
| E             | →6)-α-α-mannopyranoside (1→6) | H-2E (δ$_H$ 4.47) to C-3E (δ$_C$ 70.5); H-3E (δ$_H$ 4.33) to C-4D (δ$_C$ 73.2). C-2E (δ$_C$ 69.4); H-4E (δ$_H$ 4.48) to C-5E (δ$_C$ 71.5). C-3E (δ$_C$ 70.5); H-5E (δ$_H$ 4.29) to C-6E (δ$_C$ 67.8). C-4E (δ$_C$ 73.2); H-6E (δ$_H$ 4.28) to C-5E (δ$_C$ 71.5). C-1A (δ$_C$ 101.3) |
| F             | →3)-α-α-mannopyranoside (1→6) | H-2F (δ$_H$ 4.52) to C-3F (δ$_C$ 67.8); H-3F (δ$_H$ 4.32) to C-4F (δ$_C$ 71.2). C-2F (δ$_C$ 70.5). C-1C (δ$_C$ 103.4); H-4F (δ$_H$ 4.45) to C-3F (δ$_C$ 67.8); H-5F (δ$_H$ 4.31) to C-6F (δ$_C$ 67.8). C-4F (δ$_C$ 71.4); H-6F (δ$_H$ 4.32) to C-5F (δ$_C$ 73.2). |

Memmert, Germany). After incubation, the cultures were added to TCA to obtain a final concentration of 30% TCA and kept at 4 °C overnight. Cells and precipitated proteins were removed by centrifugation (12,000 g, 10 min, and 4 °C) in a centrifuge model K2015R. The supernatant was diluted with 99% ethanol at a 1:2 ratio. The precipitates of EPS were collected by double centrifugation in a centrifuge model K2015R and dissolved in distilled water at room temperature for further analysis. EPS yields (expressed as mg/L) were estimated in each sample by a phenol–sulfuric method using glucose as the standard.26

4.4. Production and Purification of EPS. EPS samples used for analysis of molecular weight and structural determination were obtained as described before (section 2.3) with some modifications to enhance the EPS synthesis ability of *L. plantarum* W1. The medium for producing the EPS was MRS with 5% lactose and 0.3% yeast extract, whose pH was adjusted to 6. Incubation temperature was 40 °C, and time was 36 h (UNSS, Memmert, Germany). The cultures were then added to TCA to obtain a final concentration of 20% TCA and centrifuged (13,000 rpm, 10 min, and 4 °C) in a centrifuge model K2015R to remove cells and proteins. One unit volume of the supernatant containing EPS was added to one unit volume of ethanol (99%) for collecting crude EPS.

The EPS purification was performed by eluting crude EPS solution (10 mL, 10 mg/mL) in the DEAEl-cellulose-S2 anion exchange chromatography column (particle size, 26 nm × 500 mm; rate, 1 mL/min; elution solvent: deionized water and 0.1, 0.3, and 0.5 M NaCl solutions). Four fractions were obtained. The existence of carbohydrates in the fractions was conducted via a phenol–sulfuric acid protocol. The largest water-eluted fraction, which contained 95% of neutral carbohydrates, was further isolated with the Sephadex G-100 chromatographic column (particle size, 10 mm × 600 mm; rate of 0.2 mL/min; solvent system: deionized water and 0.1 and 0.3 M NaCl solutions). Dialyzing and lyophilizing treatments of the EPS fractions from the Sephadex G-100 column were also conducted for purified EPS samples before further analysis. The fraction in which molecular weight was lower than the other fractions was selected for structural analysis.37

4.5. Phenylalanyl-tRNA Synthase (pheS) Gene Sequencing. 4.5.1. The Preparation of DNA Extracts. The alkaline lysis buffer protocol was utilized to extract the genomic DNA with some modifications.27 Briefly, the sample-derived colony was incubated in a 1.5 mL tube containing 20 μL of alkaline lysis buffer, which was obtained by mixing 0.05 mol L⁻¹ NaOH and 0.25% SDS at 95 °C for 15 min. The tube was then placed on ice before the addition of 180 μL of Milli-Q water followed by centrifugation at 13,000 rpm for 3 min. The resulted sample was stored at −20 °C for further analysis.

4.5.2. PCR and Sequencing. PheS-21-F (‘5’-CAYCNCCHCGGYAGATGC-3’) (forward primer) and pheS-23-R (‘5’-GGRGTRACCATVCCNGCHCC-3’) (reverse primer) were employed as the set of primers for PCR amplification. Typically, the composition of 16.5 μL of sterile Milli-Q water, 2.5 μL of PCR buffer 10X, 2.5 μL of dNTPs, 0.25 μL of the forward primer (50 μM), 0.25 μL of the reverse primer (50 μM), 0.5 μL of AmpliTaq DNA polymerase (1 U/μL), and 2.5 μL of alkaline lysis DNA was used for each sample. The PCR was conducted with a Veriti thermal cycler (Life Technologies) associated with a thermal program as follows: (i) 5 min at 95 °C; (ii) 3 cycles of 1 min at 95 °C, 2 min 15 s at 50 °C, and 1 min 15 s at 72 °C; (iii) 30 cycles of 35 s at 95 °C, 1 min 15 s at 50 °C, 1 min 15 s at 72 °C; and (iv) a final 7 min at 72 °C. The resulted PCR products were
interpreted through RESult 1% LE Agarose (Biozym, The Netherlands) gel electrophoresis to confirm LAB.\textsuperscript{28}

The products signaling positive PCRs were purified through the NucleoFast 96 PCR clean-up membrane system (Machery–Nagel, Germany) to collect the desired size. Such samples were filtered via ultrafiltration membranes using a vacuum pressure of up to \(-0.6\) bar to remove contaminants (e.g., primers, dNTPs, and salts). The remained PCR products on the membrane were washed with 100 μL of sterile Milli-Q water and filtered. For recovery, 70 μL of sterile Milli-Q water was utilized to eluate the PCR products, which were then used for sequencing. Subsequently, a mixture of purified and eluted PCR products (3.0 μL) was combined with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Mix version 3.1 (4 μL; Applied Biosystems, USA), 4 μM sequencing primer (3.0 μL), 56 dilution buffer (1.5 μL), and Milli-Q water(1.5 μL). PheS-21-F and pheS-23-R are the utilized primers for this sequencing. The thermal program was set up as follows: 30 cycles of 15 s at 96 °C followed by 1 s at 35 °C and 4 min at 60 °C. Afterward, the sequencing products were purified with a BigDye XTerminator Purification kit (Applied Biosystems, Life Technologies). DNA fragments were detached by an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems, USA). The injection time and voltage values were 20 s and 1.25 kV, respectively. Each run was conducted at 50 °C for 6500 s at 0.1 mA and 12.2 kV.

4.5.3. Sequence Analysis. The BioNumerics 7 software (Applied Maths) was used to analyzed the produced electropherograms. Sequences were identified by two reads for the pheS gene, and NCBI BLAST (www.ncbi.nlm.nih.gov/BLAST) was used for analyzing the pheS gene sequences.

4.5.4. Molecular Weight Determination of EPS. The average molecular weight (Mw) of EPS was determined by gel permeation chromatography (GPC) using an HPLC-UV system (Agilent 1100 Series coupled to an IR and UV detector) with an Ultrahydrogen 500 column connected in series. EPS (1 mg/mL) was eluted using 0.1 M NaNO₃ and operated isocratically at a flow rate of 1 mL/min. The injection volume was 20 μL.\textsuperscript{12}

4.5.5. GS–MS Method (Monosaccharide Composition and Methylation Analysis). Monosaccharide composition and

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**Figure 7.** 1H. 1H NOESY spectrum of EPS-W1 ((A) over; (B) expand).

**Figure 8.** Structure of the repeating units of EPS-W1, (A) α-D-glucopyranoside-(1→6)-α-D-mannopyranoside-(1→, (B) →6)-α-D-glucopyranoside-(1→), (D) →3)-α-D-glucopyranoside-(1→, (E) →6)-α-D-mannopyranoside-(1→, (F) →3)-α-D-mannopyranoside-(1→).
methylation analysis of EPS was performed according to Wang et al. and Ciucanu and Kerek with some modifications.\textsuperscript{1,2,5,6} Permethylation of EPS-W1 was carried out using NaOH-(CH\textsubscript{2})\textsubscript{3}SO-(CH\textsubscript{2})\textsubscript{3}SO\textsubscript{4}. The permethylated derivatives (5 mg) were hydrolyzed with 4 mL of 2 M TFA at 120 °C for 2 h followed by evaporation to dryness. The residue was successively reduced with excess NaBH\textsubscript{4} and acetylated with anhydride acetic/pyridine (1:1 v/v) solution at 100 °C in 20 min.\textsuperscript{31–34} The methylated alditol acetate sugars were analyzed by using the GC–MS Shimadzu system (2010) with a DB-5 capillary, with helium as the carrier gas and a linear velocity of 2 mL/min. The temperature program used was an initial temperature of 65 °C in 1 min, increasing by 8 °C/min to 250 °C and staying in 10 min, and increasing by 2 °C/min to 280 °C and staying in 5 min. The total time of the thermal program was approx. 54 min. Partially methylated alditol acetates of Man and Glc were distinguished by the NIST 14 mass spectral library and retention time.

4.5.6. Nuclear Magnetic Resonance (NMR) Spectroscopy Analysis. Twenty milligrams of EPS was hydrolyzed partially by using 5 mL of 2 M TFA at 80 °C for 9 h in a water bath (VNB22, Memmert, Germany) and then lyophilized. Ten milligrams of hydrolyzed EPS was dissolved in 10 mL of D\textsubscript{2}O and analyzed. A Bruker AM500 FT-NMR spectrometer operating at 500 and 125 MHz was used to record the \textsuperscript{1}H and \textsuperscript{13}C NMR spectrum of the polysaccharide solution, respectively. The operating temperature was 353 K. The delay (Dl) and acquisition time (AQ) were 1.00 and 3.28 s for \textsuperscript{1}H NMR spectra and 2.0 and 1.1 s for \textsuperscript{13}C NMR, respectively. The 2D spectra (HSQC, HMBC, and NOESY) were used to determine the sugar residues. Chemical shifts were expressed in parts per million (ppm).

4.6. Statistical Analysis. Data were analyzed via the one-way ANOVA method of SPSS (version 20.0) and represented as mean ± SD. The differences among procedures were tested with the Student Newman–Keuls test. The experiments were repeated 3 times. Data were considered statistically.

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**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This research was supported by the National Research Foundation of Korea (NRF) (NRF-2020R1A2C2100670) and the Korea Research Fellowship Program through the NRF funded by the Ministry of Science and ICT (grant number 2020H1D3A1A04081409). T.H.C.N. was funded by the Vingroup Joint Stock Company and supported by the Domestic Master/PhD Scholarship Programme of the Vingroup Innovation Foundation (VINIF), Vingroup Big Data Institute (VINBIGDATA). We would like to thank the Erasmus Mundus project that supported an academic staff scholarship for working at the Laboratory of Microbiology at Ghent University, Belgium.

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