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Research Article

Structural Characterization of Functional Ingredient Levan Synthesized by *Bacillus siamensis* Isolated from Traditional Fermented Food in Thailand

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The rising global population continues to threaten the world's food security. The discovery of new technologies to produce food of nutritional and functional properties is urgently needed. One beneficial food to humans of known nutritional value is the prebiotic levan. To address the problem, the present work is aimed at isolating levansucrase enzyme-producing microorganisms from traditional fermented food in Thailand. Bacterial colony morphology was observed for mucoidal consistency on culture plates. Isolated colonies were characterized morphologically by gram staining methods. Dinitrosalicylic acid (DNS) and thin-layer chromatography (TLC) reported the highest microbial enzyme activity of 8.51 IU/ml at 12 hours via hydrolysis and frutotransferase activities. Structural characterization of levan via Fourier-transform infrared spectroscopy (FTIR) and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy showed β-(2,6)-fructofuranose linkages. The highest enzyme activity was exhibited by bacterium B-6 identified as *Bacillus siamensis* NR 11274.1 based on the 16s rDNA gene sequence analyses. Thus, the isolated bacterium from the traditional food was confirmed to produce levansucrase enzyme of high industrial importance for the synthesis of levan as a functional food.

1. Introduction

Exopolysaccharides (EPSs) have been widely utilized in the food industries and dairy products as functional ingredients due to their multifunctional properties as viscosifying [1], stabilizing [2], emulsifying [3], gelling [4], and water-binding agents [5]. EPSs also were found to exhibit various health benefits, for example, in the reduction of cholesterol levels [6], inhibition of biofilm formation by pathogenic organisms [7], and as prebiotics for promoting the growth of microbiota in the human gastrointestinal tract [8].

Dietary prebiotics is a selectively fermented ingredient that stimulates specific changes in the composition and activity of the gastrointestinal microbiota and is known for many beneficial effects to the host [9]. The consumption of prebiotics has been reported to improve health by metabolizing the products by the intestinal microbiota in the likes of *Bifidobacteria* and *Lactobacilli* species [10, 11]. These microbiota consume the EPSs to synthesize several products such as short-chain fatty acids [12], lactic acid, and peptides [13]. The metabolized products are found to possess various beneficial effects in the intestinal environment, such as in

maintaining normal luminal pH in the colon, in growth stimulation of the lactic microflora in the human gastrointestinal tract, and in the production of antimicrobials against pathogens [14, 15] [16]. It is indeed clear that the consumption of prebiotics is advantageous to human health, specifically in achieving gastrointestinal homeostasis. However, known adverse effects due to excessive consumption of prebiotics have been documented, causing flatulence, abdominal disorders, and diarrhea as these products upregulate the growth and metabolism of indigenous gut microbiota [17]. Thus, several oligo- and polysaccharides for nutrition have been studied for their prebiotic potentials [18]. The recommended intake of prebiotics to confer health benefits should be followed as in inulin (8-40 g/day, 15-64 days) or fructooligosaccharides (FOSs) (4-12.5 g/day, 8-12 days) [15, 19]; both are polysaccharides found in fruits and vegetables.

Fructooligosaccharides (FOSs), fructans, β-(2-1)-inulin type, and β-(2-6)-levan type are the types of prebiotics that have been reported to be valuable in maintaining intestinal health [20]. Inulin and levan, for instance, are comparable to the prebiotic properties of fructans, which in some studies revealed that levan significantly increased the total number of microbiota in the intestine of animals [21] [22, 23]. Furthermore, numerous studies have reported other properties of levan including antitumor activity [24, 25], anti-irritant [26], antioxidant, and anti-inflammatory properties [27]; and promotion of blood clotting [28]. Moreover, levan as a nanoparticle has gained prominence for enhancing the delivery of peptides and other protein-based drugs [29, 30].

Levan structure includes the D-fructofuranosyl residues joined by β-(2-6) linkages as the main chain with some β-(2-1)-linked branched chains [31]. The degree of polymerization (DP) is estimated to be over 100,000 DP [21]. This polysaccharide is usually synthesized by levansucrase enzyme (EC 2.4.1.10), sucrose-6-fructosyltransferase, or fructosyltransferase belonging to glycoside hydrolase family 68 (GH68) produced by some strains of bacteria [32, 33]. The mechanism of levansucrases acts on catalyzing sucrose by fructosyltransferase reaction and by hydrolysis to form levan [34, 35]. The active site of levansucrases consists of 2 subsites which are -1 subside to be of high affinity for fructose residue binding and+1 subside, which is able to accommodate glucose and fructose residues. First, sucrose occupies the -1 subside covalent fructosyl enzyme, and sucrose is then hydrolyzed, releasing glucose. The process is then followed by another fructose residue on sucrose, attracting at the +1 subsite to bind the fructosyl enzyme by the fructosyltransferase reaction leading to synthesized β-(2,6)-linked oligofructans, which leads to the formation and elongation of levan [20]. Levansucrase has been known to be synthesized by several microorganisms, including the gut microbiota, for example, *Lactobacillus johnsonii*, *Lactobacillus gasseri*, *Bacillus subtilis* [36], *Aerobacter levanicum*, and *Streptococcus salivarius* [28, 33]. However, limited attempts were made to determine the production of levansucrase enzymes from bacteria present in traditional foods, such as in fermented soybeans [37].

Thus, the present study reports on the screening of isolated microorganisms from a traditionally fermented food in Thailand for levansucrase enzyme production and unravels their potential for the synthesis of levan as a functional food.

Figure 1: Mixed-growth cultures of microorganisms from fermented soybean. Isolates exhibited mucoidal colonies on agar medium containing 20% *w*/*v* of sucrose incubated at 37°C for 24 hours.

2. Materials and Methods

2.1. Isolation and Identification of Levansucrase Enzyme-Producing Bacteria. The samples of the locally fermented soybean (Thua-nao) were collected from the northeastern region of Thailand (Wiang Haeng District, in Chiang Mai Province, 50350, Thailand) and were processed for the isolation of levansucrase enzyme-producing bacteria. The cultivation and isolation of bacteria were performed following serial dilution and plate methods. The suspensions were plated onto the selective medium containing 200.0 g/l sucrose, 5.0 g/l yeast extract, 10.0 g/l tryptone, 2.5 g/l K_2HPO_4, and 15.0 g/l agar, incubated at 37°C for 24 hours. The mixed culture plates were observed for colonies exhibiting mucoidal consistency and were subcultured on fresh nutrient agar (NA) plates. Gram stain method [38] was performed to characterize the isolates morphologically and to obtain data that support the identification of bacteria from the genus to species level.

The highest levansucrase enzyme-producing isolate was identified further by the molecular techniques and through analysis of the partial 16s rDNA gene sequences. The genomic DNA was extracted from freshly grown cells in NA plates for 24 hours [39]. The 16s rDNA gene sequences were amplified by PCR with the universal primers 27F (5′-AGAGTTTGA TCATGGCTCAG-3′) and 1492R (5′TACGGTTACCTTGT TACGACTT-3′), subsequently purified by the DNeasy Tissue Kit (Qiagen, Germany), sequenced, and further analyzed [40].

Table 1: Morphology and gram stain technique classification of the 9 bacterial isolates grown on solid agar medium containing 20% (*w*/*v*) of sucrose.

Isolate code	Colony morphology					Cell type	
	Size	Shape	Opacity	Margin	Surface	Gram	Shape
B-1	Large	Irregular	Opaque	Undulate	Rough	+	Bacilli
B-2	Large	Irregular	Opaque	Undulate	Wrinkled	+	Bacilli
B-3	Large	Irregular	Translucent	Undulate	Rough	+	Bacilli
B-4	Large	Round	Translucent	Entire	Wrinkled	+	Bacilli
B-5	Large	Round	Opaque	Entire	Wrinkled	+	Bacilli
B-6	Large	Round	Transparent	Entire	Smooth	+	Bacilli
B-7	Large	Round	Translucent	Entire	Smooth	+	Bacilli
B-8	Large	Round	Translucent	Entire	Rough	+	Bacilli
B-9	Small	Round	Translucent	Entire	Smooth	—	Cocci

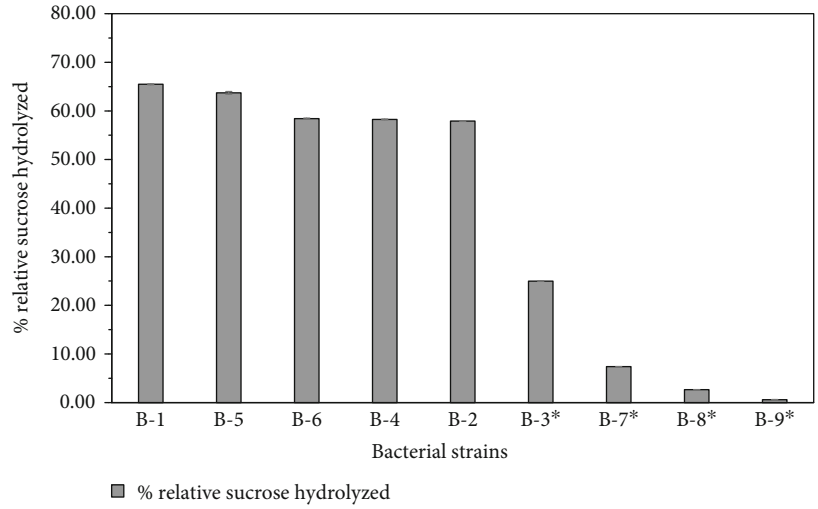

Figure 2: Results of the sucrose hydrolysis of the nine bacterial strains isolated from fermented soybean. The relative activity refers to the maximum activity. *Statistically significant difference with B-1.

PCR condition was as follows: 94°C for 3 min, 30 cycles of 94°C for 60 s, 55°C for 60 s, and 72°C for 2 min with a final extension at 72°C for 3 min. The sequence processes including the direct sequencing of 16s rDNA were performed by the Biodiversity Research Centre (BRC), Thailand Institute of Scientific and Technological Research (TISTR) (shown in Supplementary Figure 1). The partial 16s rDNA sequence was entered in the BLAST webpages of the National Center for Biotechnology Information (NCBI). Multiple sequence alignment was performed with CLUSTAL X (version 1.83) [41]. Alignment gaps and unidentified bases were edited and eliminated. The neighbor-joining tree was constructed by bootstrap analysis set to 10,000 replicates and calculated using the Kimura two-parameter method [42] with MEGA version 10 software.

2.2. Levansucrase Activity Assay

2.2.1. Production of Levansucrase Enzyme from Isolates. One loopful of the colony from the 24-hour freshly grown cultures from each isolate was inoculated into 3 ml of broth medium containing sucrose 50.0 g/l, tryptone 10 g/l, NaCl 5.0 g/l, and yeast extract 5.0 g/l, pH 7.4, in test tubes and cultivated at 37°C for 24 hours in a rotary shaker set at 200 rpm [43]. After incubation, the culture broth was observed for turbidity, and each was standardized by adding a sterile fresh broth medium to match 0.5 McFarland equivalent to the absorbance of 0.08 to 0.1 at 600 nm spectrophotometrically [43]. The tubes were incubated at the same conditions previously described. Samples were collected after incubation, centrifuged at 9100 × g (15 min, 25°C), to obtain the supernatant. The supernatants were kept and set aside for evaluation of the transfructosylating activity. The reducing sugars were determined by the DNS method [44].

2.2.2. Enzyme Mechanisms. Cultivation of bacterial isolates was performed in a 250 ml flasks containing 100 ml of fermentation medium (containing sucrose 200.0 g/l, Na_2HPO_4 3.5 g/l, NaH_2PO_4 0.8 g/l, $MgSO_4$ 0.2 g/l, $NaNO_3$ 3.5 g/l, and yeast extract 5.0 g/l), added with 10% standardized inoculum, and incubated for 24 hours at 37°C on a rotary shaker at 200 rpm [43]. Experiments were carried out in triplicate.

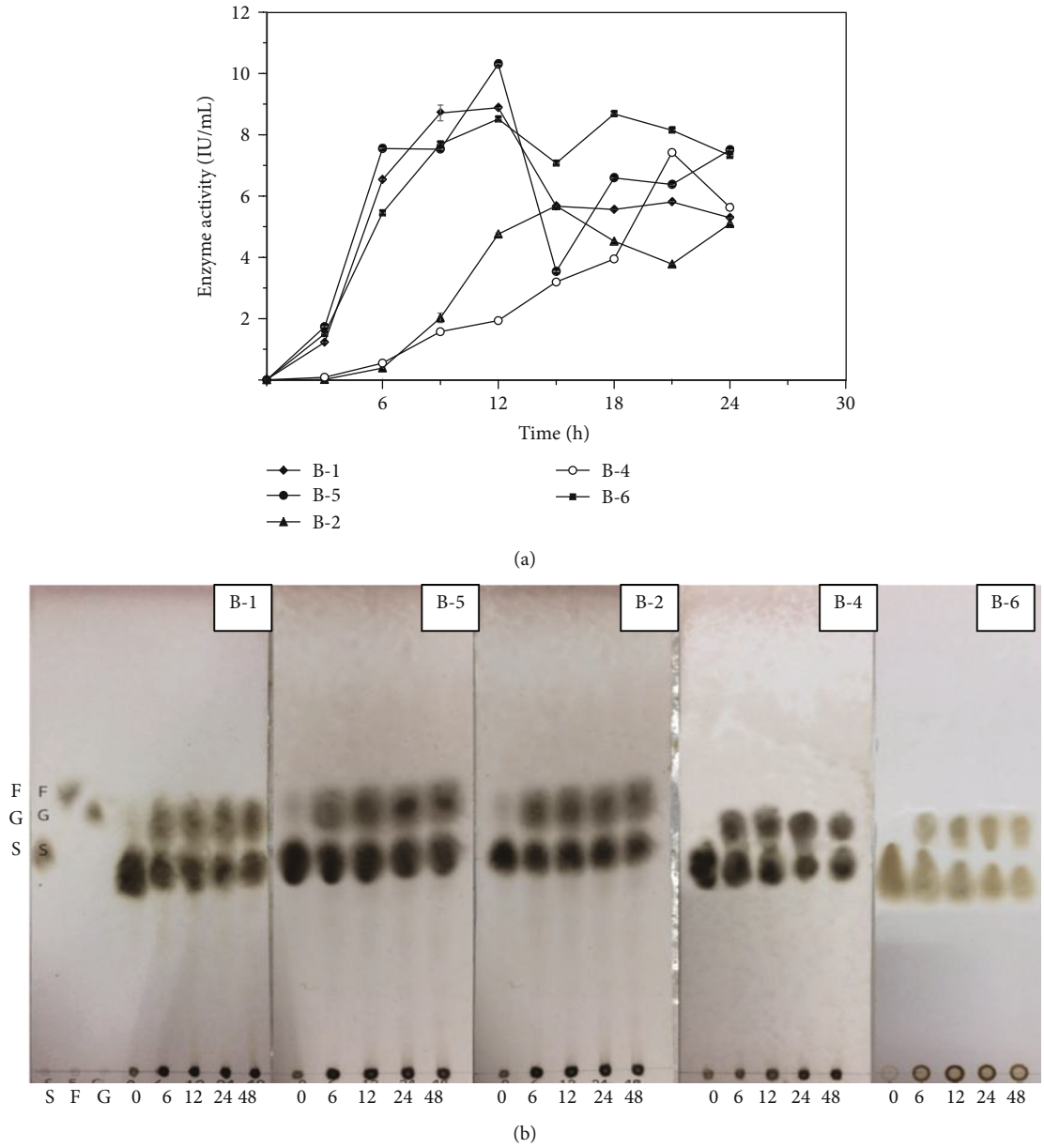

Figure 3: (a) Sucrose-hydrolyzing activity analysis of the extracellular enzyme produced by the five bacterial strains. (b) The TLC chromatogram of analyzed standard solutions: glucose (G), fructose (F), and sucrose (S); stationary phase: silica gel G-60 plates (Merck); mobile phase: chloroform/acetic acid/water (6 : 7 : 1 by volume); detection reagent: sulfuric acid and ethanol (1 : 9 (*v*/*v*)); volume of sample 10 μl; the RAF line belongs to sucrose, fructose, and glucose; 0, 6, 12, 24, and 48 line represents 0, 6, 12, 24, and 48 h; B-1, B-2, B-4, B-5, and B-6 are bacterial strains.

Sucrose hydrolysis activity was assayed by adding 0.5 ml enzyme obtained from bacterial supernatant to 0.5 ml of 20% sucrose (*w*/*v*) in 20 mM sodium phosphate buffer, pH 6.0, and the reaction mixtures were incubated at 37°C for 30 min. The reaction was stopped and the reducing sugars were determined by the DNS method. One unit of the enzyme was defined as the amount of enzyme that produced 1 μmol of glucose per minute [45].

2.2.3. Thin-Layer Chromatography (TLC) Analysis. The sugars in the reaction mixtures were analyzed by thin-layer chromatography (TLC) on silica gel G-60 using chloroform/acetic acid/water (6 : 7 : 1 by volume) as a mobile phase system. After the layer developed and the mobile phase, evaporation under continuous air for 15 min was done, and then, the mixtures were kept in an oven set at 115°C for 15 min. Spots on the chromatograms were visualized by spraying a mixture of sulfuric acid and ethanol 1 : 9 (*v*/*v*) on the TLC plate [46].

2.3. Isolation, Purification, and Identification of Levan

2.3.1. Isolation and Purification of Levan. Cultivation was performed in flasks containing 200 ml of fermentation

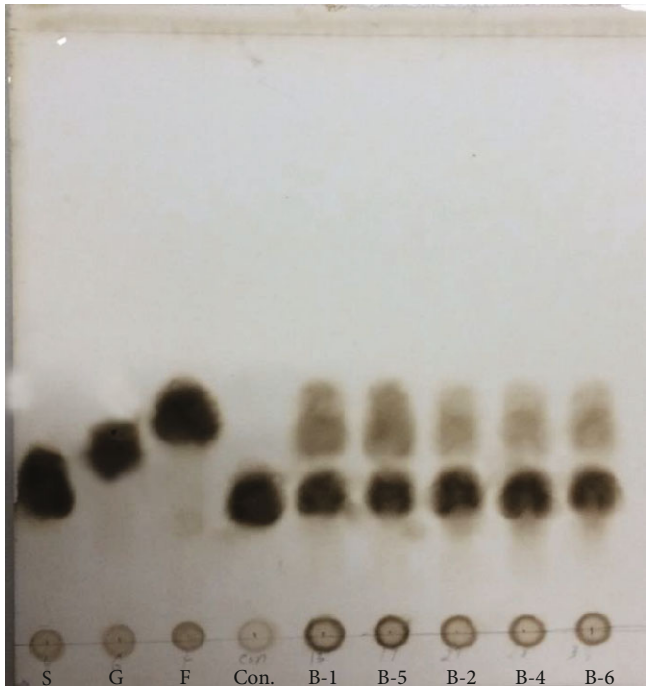

Figure 4: The TLC chromatogram of analyzed standard solutions: glucose (G), fructose (F), and sucrose (S); stationary phase: silica gel G-60 plates (Merck); mobile phase: chloroform/acetic acid/water (6 : 7 : 1 by volume); detection reagent: sulfuric acid and ethanol (1 : 9 (*v*/*v*)); volume of sample 10 μl; the RAF line belongs to sucrose, fructose, and glucose; B-1, B-2, B-4, B-5, and B-6 are bacterial strains.

medium and inoculated with 10% standardized inoculum and incubated for 3 days at 37°C on a rotary shaker set at 200 rpm. Cell removal by centrifugation at 4500 rpm (45 min, 4°C) was done, and the supernatant was preliminarily analyzed and identified by thin-layer chromatography (TLC) for its compound.

The supernatant was mixed with absolute cold ethanol in a ratio of 1 : 3 (*v*/*v*). The reaction mixture was incubated at -20°C for 24 hours. The reaction mixture was subsequently centrifuged at 4500 rpm for 45 min at 4°C to collect the precipitate. This process was repeated until no leftover was observed from the original mixture. The pellets were dissolved in hot water followed by deproteinization by the Sevag method by adding Sevag reagent (n – butanol : chloroform = 1 : 5, *v*/*v*) to the reaction mixture and to remove denatured protein which appeared at the interface between water and chloroform layers. The procedure was repeated five times to increase yield. The supernatant was subjected to dialysis with a bag with an MWCO of 12,000 Da against deionized water at 4°C for five days [47]. Consequently, deionized water was replaced with fresh water and the crude levan was obtained by lyophilization [48].

2.3.2. Fourier-Transform Infrared Spectroscopy (FTIR) Analysis. Fourier-transform infrared spectroscopy (FTIR) analysis was conducted for the determination of the functional group deposition of the structure of levan. The FTIR spectra were recorded in transmittance mode from the wavenumber of 4000 to 400 cm^{-1} on an FTIR spectrophotometer (Perkin Elmer, Frontier) at the Department of Chemistry, Faculty of Science, Burapha University, Thailand.

2.3.3. ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) Spectroscopy. The levan structure was analyzed using NMR spectroscopy. Here, 0.5 mg of levan was dissolved in D_2O for each of ^{1}H NMR and ^{13}C NMR analysis. Then, the sample was submitted to Heteronuclear Single-Quantum Correlation (HSQC) analysis using D_2O solvent. Spectra were recorded on Bruker UltraShield 400 MHz/54 mm bore. ^{1}H NMR spectra and ^{13}C NMR spectra were run at 400 MHz. (Bruker, AVANCE UltraShield 400 MHz spectrometer) at the Department of Chemistry, Faculty of Science, Burapha University, Thailand.

3. Results and Discussion

3.1. Isolation and Identification of Levansucrase Enzyme-Producing Bacteria. The bacteria isolated from the fermented soybean for the screening of levansucrase enzyme exhibited mucoidal colonies grown on solid medium. This colonial characteristic may be due to their production of extracellular levansucrase enzyme activities that converted sucrose present in the medium to exopolysaccharides in the form of β-d fructoside (levan) and glucose released. Figure 1 shows the colonies of the bacterial strains with mucoidal consistency. Thus, the bacteria exhibiting such consistency were the basis for the isolation of the bacteria for further analysis. The bacteria were subcultured onto the fresh solid medium by a standard technique. From the forty-three (43) grown colonies in the mixed culture plates, nine (9) of them exhibited discreet and mucoidal consistency. The isolates were further characterized by staining methods and found to be gram-positive, rod-shaped bacteria and have one gram-negative coccus (Table 1). The biosynthesis of levansucrase enzyme by the isolates on the selective medium containing high sucrose may be explained by the bacterial excretion mechanisms commonly observed in both gram-positive and gram-negative bacteria. Levansucrase enzyme biosynthesis in gram-positive bacteria includes enzyme accumulation into the periplasmic space and by the excretion of the enzymes out of the cell into the surrounding environment by the cleavage of a signal peptide, whereas in gram-negative bacteria, the signal peptide pathway initiates the secretion of levansucrase into the outer environment [49].

3.2. Levansucrase Activity Assay

3.2.1. Production of Levansucrase Enzyme from Isolates. The isolated bacterial strains selected following colony morphology and gram stain techniques were further evaluated for levansucrase enzyme production and activity. The result reveals that out of the nine bacterial strains, five strains (B-1, B-5, B-6, B-4, and B-2) showed potential for EPS production and selected for extracellular production of the enzyme (Supplementary Figure 8). The extracellular levansucrase enzyme produced by the isolates was estimated by the

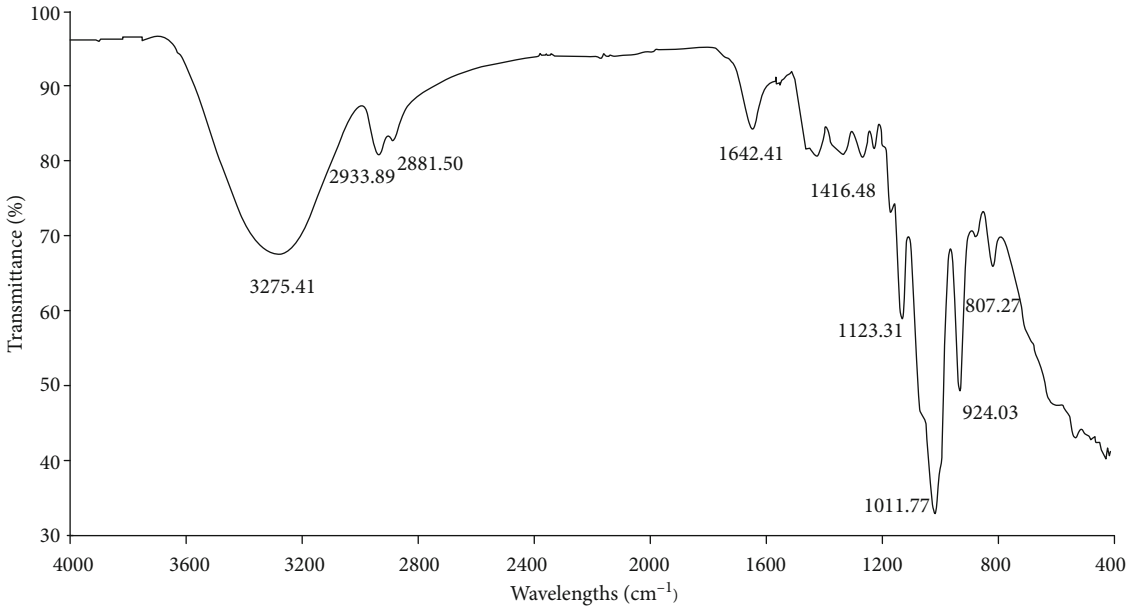

Figure 5: The FTIR spectrum of the produced levan from B-6 bacterial strain.

percentage efficiency of sucrose hydrolysis and calculated from the concentration of reducing sugars released (Supplementary Table 1), wherewith the mechanism of levansucrase synthesized polysaccharides and released reducing sugar from the reaction; thereby, we assume that the increasing concentration of reducing sugar is associated with high enzyme activity. Figure 2 shows the performance of the enzyme activities of the nine bacterial strains. The activity exhibited by the five strains was capable of hydrolyzing sucrose for as high as 50%. On the other hand, the other four bacterial strains (B-3, B-7, B-8, and B-9) exhibited less than 59% sucrose hydrolysis. The percentage of sucrose hydrolysis of the top 5 performing enzyme-producing bacterial strains is significantly different from the enzyme activity of the four bacterial strains. Thus, isolates B-1, B-5, B-6, B-4, and B-2 were selected for further enzyme analysis. The present findings on extracellular production of enzyme by microorganism agree with the study of Maugeri and Hernalsteens [45] on the screening of the microbes obtained from the fruits and flowers that were found to secrete extracellular enzymes with high fructosyltransferase activities. The same standard medium previously described for enzyme activity consisting of 2% yeast extract, 5% sucrose, 1% $NaNO_3$, 0.01% $MgSO_4{\cdot}7H_2O$, and 0.1% K_2HPO_4 (pH 5.5) was utilized where 495 yeast strains and 130 bacterial strains (about 25% of the isolated strains) showed high hydrolysis activity under the sucrose-containing medium comparable to the results presented hereof [45].

3.2.2. Enzyme Mechanisms. The five enzyme-producing bacteria were recultured for the production of extracellular enzymes in the fermented medium. Enzyme assays were determined in a reaction containing 20% (*w*/*v*) sucrose in 20 mM sodium phosphate buffer, pH 6.0, mixed with the crude enzyme. As shown in Figure 3(a), B-5, B-1, and B-6 have predominantly exhibited the highest enzyme activity of 10.30 IU/ml, 8.88 IU/ml, and 8.51 IU/ml at 12 h of fermentation, respectively. Isolate B-2 has shown the highest enzyme activity at 15 h (5.68 IU/ml), and isolate B-4 has the highest activity at 21 h (7.41 IU/ml). Initially, a high concentration of sucrose in the medium activated and induced the levansucrase production and persisted at the stationary phase of growth. At this phase, the enzyme activity was gradually decreasing as the concentration of sucrose also decreased. The study of Li [20] explained that the levansucrase enzyme activity was dependent on the initial substrate concentration where the microorganisms are grown. The high concentration of substrate activates enzyme production, thus triggering the enzyme release extracellularly by the microbe. This activity was observed to be at the maximum concentration at the stationary phase of growth, following a decline of the activity at the death phase. The sucrose hydrolysis activity in the study is similar to the activity of *B. amyloliquefaciens* isolated from maple syrup (30°Bx) at 48 h which resulted in the highest levansucrase activity (427.53 μmol/mg protein/min) [20]. Moreover, Dajanta et al. [37] reported that during the growth phase, microbes synthesize proteolytic enzymes to digest the free amino acids present in the substrate. These findings strongly support the premise that the production of enzymes is at the maximum during the initial growth of organisms grown in high-substrate-containing medium.

3.2.3. Thin-Layer Chromatography (TLC) Analysis. After evaluating the mechanisms of the enzymes exhibited by the bacterial strains, their mixed solutions were spotted on TLC plates to determine the sugar component. Figure 3(b) shows the enzymatic reaction of all bacterial strains to hydrolyze sucrose and covert monosaccharide residue to oligosaccharides and polysaccharides. The results show that there is

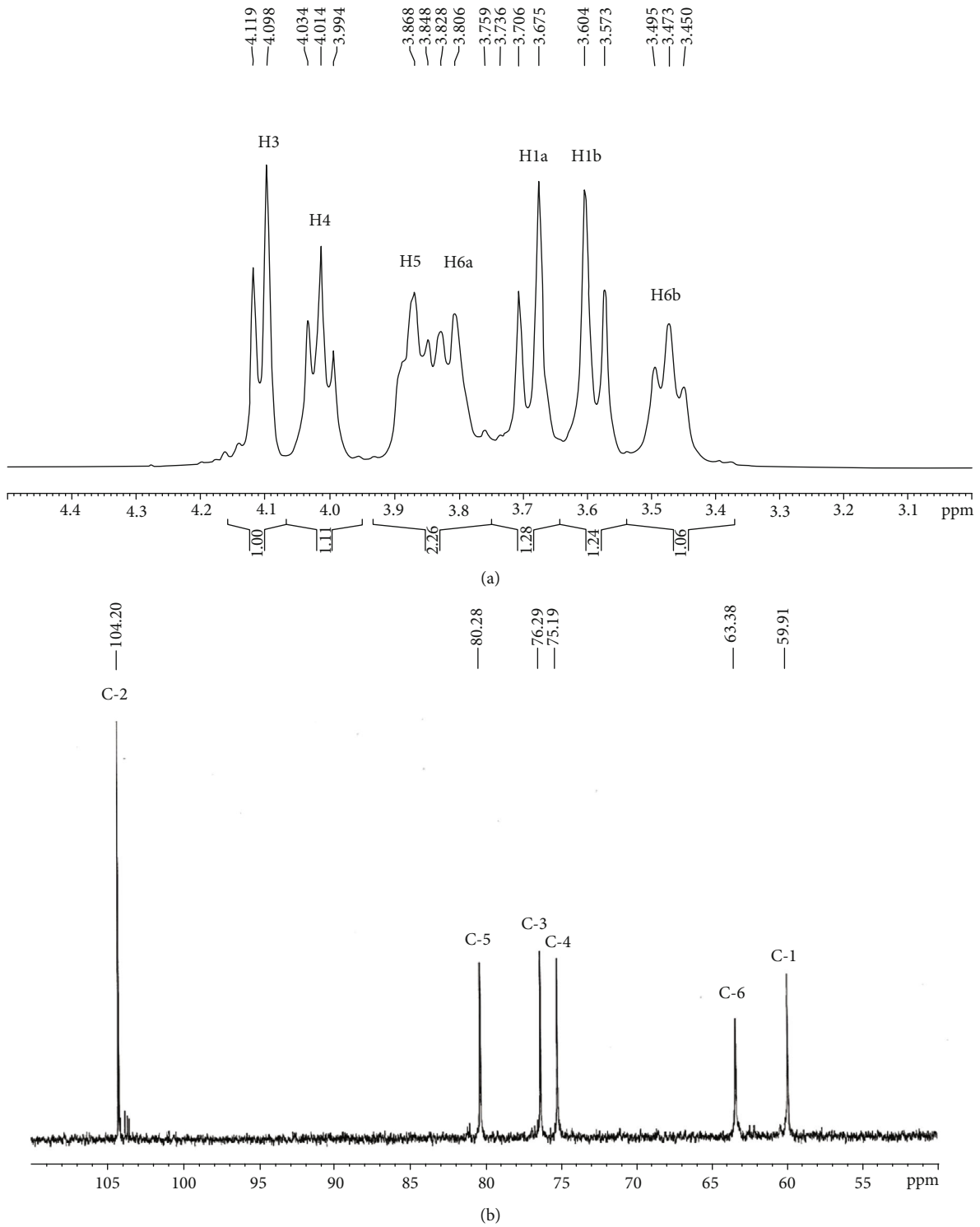

Figure 6: (a) The identification of levan produced from levansucrase of B-6 bacterial strain by ^{1}H NMR spectrum levansucrase. (b) The identification of levan produced from levansucrase of B-6 bacterial strain by ^{13}C NMR spectrum levansucrase.

one spot of sucrose at lines 0 (at initial culturing) because the bacteria are adapting to the new environment as they divide during the lag phase. At lanes 6, 12, 24, and 48 (at 6, 12, 24, and 48 hours of culturing time) as log phase and stationary phase of cell growth progress, all bacteria grow and produce enzymes for the production of EPS. For this reason, the sugar spot at lanes 6, 12, 24, and 48 revealed two parts on the TLC plate: the top part as monosaccharides and disaccharides, both of which were hydrolyzed by the extracellular enzyme, and the bottom part as EPSs that was generated from monosaccharide residues by transferase activity. TLC analysis may have limitations to distinguishing the molecular weight of

Table 2: The comparison of ^{13}C chemical shift signals of levan produced by bacterial strain B-6 and by other bacteria reported elsewhere [34, 36, 41].

Carbon atom	The chemical shift (ppm) of the levans formed from			
	B-6[a]	*B. megaterium* GJT321 [36]	*B. licheniformis* BK AG21 [34]	*B. methylotrophicus* [41]
C-1	59.91	59.86	59.81	61.20
C-2	104.20	104.14	104.22	104.66
C-3	76.29	76.25	76.24	77.51
C-4	75.19	75.31	75.18	76.10
C-5	80.28	80.23	80.31	80.77
C-6	63.38	63.31	63.40	65.69

[a]Bacterial strain in this study.

polysaccharides higher than 10 DP (degree of polymerization); thus, the extracellular enzyme catalyzed sucrose to the high molecular weight [50]. However, this method is aimed primarily at analyzing enzyme activity and at characterizing the enzymatic reaction. The EPSs produced from all the bacterial strains were isolated, and their structure was identified through the analytical methods.

3.3. Isolation, Purification, and Identification of Levan

3.3.1. Isolation and Purification of Levan. The EPSs produced by the extracellular enzyme from 5 bacterial strains in the fermented medium were characterized by TLC analysis using mono- and disaccharide detection (Figure 4). The results showed that the enzyme activity of the three bacterial strains, B-2, B-4, and B-6, synthesized fructans shown at a bottom spot by fructotransferase activity along with the hydrolysis activity exhibited in the spots of glucose and fructose. We compared the intensity of both spots and found that the concentration of fructose is lower than that of glucose as fructose residues were catalyzed by the fructotransferase activity into a fructan form. This activity was displayed at the lowest spots on the chromatogram as a fructan of high DP. The findings adhere with the report of Watanabe and Oda [51] on *A. rouxii* CBS 438.76 which hydrolyzed the FOSs, inulin, and levan into fructose, completely [51]; however, fructan production was generated by linking fructose residue of sucrose and releasing glucose, thus resulting to higher glucose concentration than fructose.

The three bacterial strains, B-2, B-4, and B-6, based on their previous enzyme activities and TLC analysis were further investigated. The enzyme activities of B-6 bacterial strain were found to be at the highest performance at 8.51 IU/ml at 12 h. The fructotransferase activity of B-6 bacterial strain was catalyzed to produce fructans into promising a levan form. The biosynthesis of fructan was confirmed with the cultured B-6 bacterial strain through EPS purification process. FTIR and NMR spectroscopies further characterized the product.

3.3.2. Fourier-Transform Infrared Spectroscopy (FTIR) Analysis. FTIR analysis was conducted for the determination of the functional group deposition of the structure for levan production for B-6 bacterial strain and was recorded in transmittance mode from the wavenumber of 4,000 to 400 cm^{-1} and was compared to the standard levan (Supplementary Figures 6–7). Figure 5 presents a broad and strong peak at 3600-3200 cm^{-1} (specifically, at 3275.41 cm^{-1}) as O-H stretching vibration due to intermolecular hydrogen bonding [52]. C-H stretching vibration appears at 3,000-2,800 cm^{-1} (specifically, at 2933.89 and 2881.50 cm^{-1}). The broad peak at 2933.89 cm^{-1} is the methylene group that vibrated asymmetrically, but the broad peak at 2881.50 cm^{-1} vibrates symmetrically. C-H bending vibration of the methylene group appears at 1416.48 cm^{-1} [53]. A broad band at 1642.41 cm^{-1} was due to the bound water [54]. C-O-H stretching vibration appears at 1123.31 cm^{-1}, and the strong broad peak at 1011.77 cm^{-1} was characterized by glycosidic linkage (C-O-C) stretching vibration in pyranose or furanose which is the carbohydrate fingerprint [55, 56]. The broad peak at 924.03 and 807.27 cm^{-1} is confirmed as the furanose of the sugar units [57]. The spectra show two broad peaks at 917 and 770 cm^{-1}, characteristic of several pyranoses [58]. The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of polysaccharide. These values are almost identical to the previous report on the values of levan produced by *Brenneria goodwinii* [48].

3.3.3. Analysis of ^{1}H, ^{13}C NMR, and 2D Heterocorrelated HSQC Spectra. ^{1}H and ^{13}C NMR spectroscopy provides complementary data regarding the levan structure. The ^{1}H NMR spectra (Figure 6(a)) were also observed for the proton chemical shift signal related to the fructose as a monomer of levan. This signal which was present at the chemical shift of 4.098 ppm (H3), 4.014 ppm (H4), 3.867 ppm (H5), 3.828 ppm (H6a), 3.673 ppm (H1a), 3.604 ppm (H1b), and 3.48 ppm(H6b) (shown in Supplementary Figures 2–4) corresponded to the levan product resonance of *B. megaterium* GJT321 [55], *B. methylotrophicus* SK 21.002 [47], and *B. licheniformis* BK AG21 [53].

The ^{13}C spectrum (Figure 6(b)) showed six resonance signals, 104.02 ppm (C2), 80.28 ppm (C5), 76.29 ppm (C3), 75.19 ppm (C4), 63.38 ppm (C6), and 59.91 ppm (C1) (shown in Supplementary Figure 5), which confirmed hexose shape of the monosaccharides of EPS and are related closely to levan since these were identified by the carbon chemical shift of levan that has been reported from the previous studies (Table 2).

Additionally, the six signals displayed from ^{13}C NMR spectral analysis correspond to the fructose residue. The anomeric carbon atom (C2) was shown at 104.02 ppm while C1 and C6 of the methylene group was shown at 59.91 and 63.38 ppm, respectively. The peak signal at 80.28 ppm was attributed to furanose (C2), whereas the peaks at 76.29 and 75.19 of C3 and C4, respectively, correlated to oxymethinic groups.

Furthermore, the HSQC analysis was performed to investigate hydrogen atoms linked to their respective carbon atoms. The obtained spectra displayed the corresponding cross-peaks between H1a/C1, H1b/C1, H3/C3, H4/C4, H5/C5, H6a/C6, and H6b/C6. However, no cross-peaks at C2 were observed (Table 3). The results affirmed the quaternary anomeric

Table 3: The HSQC chemical shifts signals in D_2O of the fructose residue of levan product from B-6 bacterial strain.

	The chemical shift (ppm)							
Atoms	H1a/C1	H1b/C1	C2	H3/C3	H4/C4	H5/C5	H6a/C6	H6b/C6
^{1}H	3.61	3.72	—	4.11	4.05	3.87	3.82	3.52
^{13}C	60.0	60.0	104	76.2	74.1	79.0	63.0	63.0

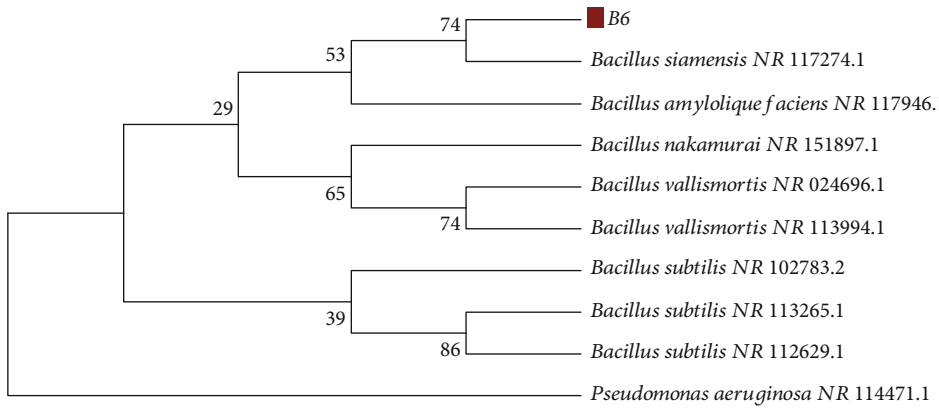

Figure 7: Neighbor-joining tree constructed in comparison with the 16s rDNA gene sequences of the isolated levansucrase-producing bacteria with sequences available in GenBank. Bacterial strain B-6 has high similarity with *B. siamensis* NR 11274.1. *P. aeruginosa* NR 114471.1, a gram-negative bacterium, was used as an outgroup.

carbon character (C2). This evidence shows that the signals from the ^{1}H, ^{13}C NMR, and the HSQC refer to the characteristics of β-(2,6) linkages of the fructose residue of the levan product from levansucrase of the B-6 bacterial strain.

3.4. Bacterial Identification. Based on the overall activity of all the enzyme-producing bacteria isolated in this work, the B-6 bacterial strain was the main interest for its highest levansucrase enzyme production for the synthesis of levan. The morphological characteristics of the colony of the isolate were large, round, transparent colonies, with a smooth surface. Microscopically, the isolate appeared as gram-positive and rod-shaped. Based on the molecular analysis of the strains, 16s rDNA gene sequences demonstrated the highest percent similarity of 99.68% with *B. siamensis* NR_117274.1 (a partial sequence comprising 1,525 bp) available in GenBank. Figure 7 shows the neighbor-joining tree constructed with the gene sequence of the strain of interest with those sequence results from the BLAST database. The BLAST results were utilized for the molecular identification of levansucrase enzyme-producing bacteria in the study. A similar study reported the isolation of gram-positive, rod-shaped levansucrase enzyme-producing microorganism with identity belonging to *B. methylotrophicus* based on the 16s rDNA gene sequences analysis [47].

4. Conclusions

The results from this study demonstrated the presence of levansucrase enzyme-producing bacteria isolated from the traditional Thai fermented food and their potential application for production of the prebiotic levan. The screening and isolation procedures for extracellular levansucrase activity resulted in five bacterial strains (B-1, B-2, B-4, B-5, and B-6) exhibiting varying sucrose hydrolyzing activities. Isolates B-5, B-1, and B-6 showed the highest total activity at 12 hours at 20.60, 17.16, and 8.51 UI/ml, respectively. Overall, bacterial strain B-6 had the highest fructosytransferase activity for producing fructan. Based on the FTIR, NMR, and HSQC analyses, the structure for the fructan was a levan consisting of consecutive β-(2,6)-fructopyranose units. Based on the morphological and molecular identification, 16s rDNA gene sequence analysis confirmed the identity of the bacterial strain as *B. siamensis* NR 11274.1. Factors such as varying temperatures, agitations, incubation time, carbon sources, and the use of other culture media may be explored further to determine their influence in the levansucrase enzyme production of the isolates.

Data Availability

The data used to support the findings of this study are included within the supplementary information file(s).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Supplementary Figure 1: the 16s rDNA sequencing analysis results revealing the identity of levansucrase-producing bacterial isolate B-6 as *Bacillus siamensis* NR 11274.1 based on the percent (%) similarity score of 100. The results have been generated by the Thailand Institute of Scientific and Technological Research, Biodiversity Research Center, March 2, 2018. Supplementary Figure 2: the 2D NMR testing results of the levan product produced by strain B-6. The HSQC spectrum in D_2O of the levan product from levansucrase of B-6 bacterial strain displays the direct connectivity between ^{13}C and ^{1}H ($J_{H/C}$) of the fructose residue of levan (1st time). Supplementary Figure 3: the HSQC spectrum in D_2O of the levan product from levansucrase of B-6 bacterial strain displays the direct connectivity between ^{13}C and ^{1}H ($J_{H/C}$) of the fructose residue of levan (2nd time). Supplementary Figure 4: the HSQC spectrum in D_2O of the levan product from levansucrase of B-6 bacterial strain displays the direct connectivity between ^{13}C and ^{1}H ($J_{H/C}$) of the fructose residue of levan (3rd time). Supplementary Figure 5: the identification of levan produced from levansucrase of B-6 bacterial strain by ^{13}C NMR spectrum levansucrase. Supplementary Figure 6: the FTIR spectra of the reference standard levan. Supplementary Figure 7: the FTIR spectra of the levan produced by the bacterial strain, B-6. Supplementary Figure 8: results of the relative nine bacterial strains isolated from fermented soybean. The relative activity refers to the maximum activity. Isolates B-3, B-7, B-8, and B-9 are statistically different with B-1. Supplementary Figure 9: sucrose-hydrolyzing activity analysis of the extracellular enzyme produced by the five bacterial strains. Supplementary Table 1: DNS assay spectrophotometer readings of the relative sucrose hydrolysis of the nine bacterial strains isolated from fermented soybean. Supplementary Table 2: determination of the enzyme activity concentration of reducing sugar by the DNS method. *(Supplementary Materials)*

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