

Biosynthesis And Structural Characterization of Levan By A Recombinant Levansucrase From *Bacillus Subtilis* ZW019

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Abstract

Purpose: The yield of levan extracted from microbial fermentation broth is low, so in vitro catalytic synthesis of levan by levansucrase is expected to be one of the industrial production approaches of levan.

Methods: A recombinant plasmid pET-28a-AcmA-Z constructed in the previous study was used to produce levansucrase. The effects of temperature, pH, and metal ions on the levan formation activity of the levansucrase were investigated. The polymer was analyzed by means of HPIC, FTIR, NMR techniques.

Results: The recombinant levansucrase could be easily purified in one step and the purified enzyme had a single band clearly visible in SDS-PAGE. The conditions for enzymatic reactions was optimal at pH 5.2 and 40 °C, and the activity of enzymes was stimulated by K⁺ and Ca²⁺. The yield of levan biosynthesis from 10% (w/v) sucrose with 6.45 U/g sucrose of levansucrase was 30.6 g/L. The molecular weight of the levan was about 1.56×10⁶ Da, as measured by GPC. HPIC analysis showed that the monosaccharide composition of the levan was fructose and glucose. The results of FTIR and NMR analysis indicated that the polymer produced by the recombinant levansucrase was β-(2, 6) levan.

Conclusions: The results of this study provide a basis for large-scale production of levan by enzymatic method.

1. Introduction

Levan is a fructan biopolymer, consisting of repeating fructose subunits with a glucose residue at the reducing end. Levan's fructose chain is formed by β-(2,6) glycosidic bonds with some β-(2,1) linked branch chains [1–3]. Due to its excellent biocompatibility, biodegradability, renewability, eco-friendliness, and human compatibility, levan has a wide range of applications in the fields of food, pharmaceuticals and cosmetics [4, 5]. In the food industry, levan is used as a stabilizer, encapsulating agent, water-holding agent, surface-finishing agent, thickener, emulsifier, flavor carrier, and prebiotic sweetener [6]. In addition, levan can also increase the shelf life of food and be used as a fat substitute [7]. In the field of pharmaceuticals, levan can be used as a plasma substitute [4] and have anti-hyperglycemia, anti-diabetes, anti-oxidation, anti-virus [8], cholesterol-lowering [9], anti-tumor [10] and immunomodulatory bioactivities. For example, levan produced by the levansucrase from *Leuconostoc mesenteroides* NTM048 can induce IgA production in rats [11]. In the field of cosmetics, levan plays an important role in cell proliferation, skin moisturizing and skin irritation-alleviating as a mixed ingredient [1, 12], because it can protect plant cells by storing water and controlling the active ingredient in a fixed area during the growth of plant cells.

Levan is naturally produced by plants or microorganisms. Microbial levans have a much larger molecular weight (2 to 100 MDa) than plant-produced levans (2 to 33 KDa) and have more branches [13]. Due to the low content of levan in plants and the high cost of natural extraction and separation, levan is not suitable for industrial production. It is a good choice to synthesize levan by the microorganism. So far, the

production of levan is mainly biosynthesized by levansucrase using sucrose as substrate. Levansucrase catalyzes hydrolysis, transglycosylation, and polymerization reactions in the formation of levan [14]. Many microorganisms can produce levan, such as *Zymomonas* [5], *Halomonas* [15], *Bacillus* [16], *Acetobacter xylinum* [17], *Pseudomonas* [18] and *Leuconostoc mesenteroides* [19], but most of their production is too low to meet commercial needs. Therefore, levan production by enzymatic method may be an effective choice to solve the problem of low yield of wild strains.

In this study, we used the pre-constructed genetically engineered bacteria *Escherichia coli* BL21 to produce levansucrase (Genbank MT038999). The effects of temperature, pH, and metal ions on the activity of the purified recombinant enzyme were investigated to evaluate its potential to synthesize levan. The polymer was analyzed by means of high-performance ion chromatography (HPIC), Fourier transform infrared (FTIR), nuclear magnetic resonance (NMR) techniques. The structure of the biosynthetic product was determined to be levan-type fructan.

2. Materials And Methods

2.1 Materials

E. coli strains DH5α and BL21 were cultivated aerobically at 37 °C in lysogeny broth (LB). Restriction endonucleases, Prime STAR Max DNA Polymerase, and DNA Ligation Kit were obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China) or New England BioLabs (Beijing, China). The DNA primers and Plasmid Mini Kit were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). All other reagents were of analytical grade and were commercially available unless otherwise indicated.

2.2 Gene cloning, expression and purification of the recombinant levansucrase

In the previous study, we have constructed a levansucrase expression system using *E. coli* BL21 as the host [20]. The recombinant genetic engineering bacterium were harvested by centrifugation at 12,000 rpm for 20 min at 4°C, and the precipitated fractions were labeled as intracellular components. The intracellular components were washed twice with 50 mM Tris-HCl buffer (pH 7.0) and subsequently disrupted by ultrasonic oscillation. The cellular debris and unbroken cells were separated by centrifugation at 12,000 rpm for 25min at 4°C. The supernatant after centrifugation was labeled as the source of levansucrase, and the precipitate was labeled as inclusion bodies. In order to purify the protein, the Gram-positive enhancer matrix (GEM) particles were combined with the Acma-labeled recombinase, centrifuged at 4°C 12,000 rpm for 30 min to collect the precipitate and dissolved in the constant volume of Tris-HCl (pH 7.2) buffer solution [21]. The SDS-PAGE was carried out on a electrophoretic device SE 250 (GE Healthcare, USA) using a 12% separation gel and 5% stacked gels under a constant 100 mV current. Proteins in the gel were stained with Coomassie blue G-250 and destained with ddH₂O. After SDS-PAGE gel electrophoresis analysis, the correct expression of the enzyme was confirmed.

2.3 Measurement of the levan formation activity of levansucrase

In order to determine the levan formation activity, 1ml 5% phenol and 5ml concentrated sulfuric acid were added to the reaction system. The solution was placed at room temperature for 20min, and then the absorbance was measured at 490 nm by UV-VIS Spectrophotometer (MAPADA, V-1800).

2.4 Effect of temperature, pH, metal ions on the levan formation activity of the recombinant levansucrase

The optimum temperature for the activity of levansucrase was detected by cultivating the reaction mixture at 20.0–60.0°C at pH 5.2 sodium acetate-acetate buffer using sucrose as the substrate (10% w/v), and the optimum pH for enzyme activity was tested at pH ranges (4.6–5.6). The effect of metal ions was detected by preprocessing the levansucrase in the 50 sodium acetate-acetate buffer (pH 5.2) containing 5 mM or 50 mM of K⁺, Ca²⁺, Cu²⁺, Mg²⁺, Fe²⁺, Fe³⁺, Ba²⁺, Zn²⁺, Ni²⁺ and Mn²⁺ at 40°C for 2 h. All the experiments were performed in triplicate, and the error was expressed as the standard deviation of the three measurements.

2.5 Determination of kinetic parameters

Kinetic parameters of the levansucrase reactions were determined by varying the sucrose concentration (0.0078–0.125 M) at 40°C and pH 5.2. We used DNS (3,5-dinitrosalicylic acid) method to determine the released total reducing sugar concentrations [24]. Data were fitted to the standard Michaelis-Menten formula.

2.6 Preparation and purification of levan

The crude polysaccharide solution after the enzyme reaction was centrifuged at 4 °C 8,000 rpm for 30 min. The supernatant was added with three times the volume of precooled 95% (v/v) ethanol, and the polysaccharide was precipitated overnight at 4 °C. Then the polysaccharide was centrifuged according to the above conditions and redissolved in an appropriate amount of ultrapure water. The redissolved solution was dialyzed with ultrapure water at 4 °C for 72 h in a dialysis bag with a cut-off of 14 kDa, and the water was changed every 8 h. Finally, the polysaccharide sample was obtained by lyophilization.

2.7 Monosaccharide Composition Analysis

In this study, high-performance ion chromatography (HPIC, ThermoFisher ICS5000) was used to determine monosaccharide composition. The 13 kinds of monosaccharide standard (fucose, rhamnose, arabinose, galactose, glucose, xylose, mannose, fructose, ribose, galacturonic acid, glucuronic acid, guluronic acid, and mannuronic acid) were prepared into 10 mg/ml standard solution, respectively. 4 mg of the sample was put into ampoule, and 1 ml 2 mol/L trichloroacetic acid was added into it. The ampoule filled with the solution was put into an oven for hydrolysis for 2 h under the condition of 100 °C. The acid hydrolysis solution of 200 µL was transferred to a 1.5 mL eppendorf tube for nitrogen blowing

and drying, and 1mL water was added for vortex mixing. The solution was centrifuged at 12, 000 rpm for 5 min. The supernatant was added into IC for analysis. The detector used for ion chromatography analysis was an electrochemical detector. Chromatographic column type was DionexCarbopacTMPA20 (3*150) and the detection temperature was 30 °C. Mobile phase: A: H₂O; B: 250mM NaOH; C: 50mM NaOH & 500mM NaOAC. The flow rate was 0.3 ml/min and the injection volume was 5 µL.

2.8 Levan structural characterization and identification methods

2.8.1 Molecular weight and purity of levan

The average molecular weight and purity of the levan were determined by gel permeation chromatography (GPC, Waters 1515) with Waters 2414 Refractive Index Detector. The ULTRAHYDROGEL 120, 250 and 500 PKGD columns were eluted with 0.1 M NaNO₃ at a flow rate of 1 mL/min. Samples (25 mg/L, 50 µL) were injected into the column. PEG standard compounds (Mp 330000, 176000, 82500, 44000, 25300, 20600, 12600, 7130, 4290, 1400, 633, 430 KDa) were provided by Polymer Standards Service-USA Inc [25].

2.8.2 Scanning electron microscope (SEM) of levan

The surface morphology of the levan was obtained by Scanning Electron Microscope (SEM, SU8020) technique. Freeze-dried pure levan was fixed to the SEM stubs with double-faced adhesive tape and coated with conductive gold in an ion sputtering apparatus. The microstructures of samples with different magnification were observed at an accelerating voltage of 10 kV. In this study, the SEM images of levan samples at magnification 400×, 1000×, 2000× were obtained.

2.8.3 Atomic force micrograph (AFM) of levan

The surface morphology and roughness of levan were obtained by Atomic force micrograph (AFM, Bruker Multimode8) analysis. The levan solution with a concentration of 1 mg/ml was prepared and stirred continuously for 1 h at 40 °C in an airtight bottle. Once cooled to room temperature, 5 µL of levan solution was absorbed into the mica sheet. The AFM images of levan were obtained in tapping mode after drying at room temperature.

2.8.4 Fourier-transform infrared spectroscopy (FT-IR)

Fourier-transformed infrared spectroscopy is a common method for the determination of functional groups presented in levan. The levan and KBr powder were thoroughly mixed in a ratio of 1:100 to make a 1 mm thickness KBr pellet. The absorption spectrum of levan was recorded on a Thermo IS5 instrument (America) in the region of 4000 – 400 cm⁻¹.

2.8.5 Nuclear magnetic resonance spectroscopy (NMR)

¹H and ¹³C NMR spectrum of the levan solution were recorded using a Bruker 600M spectrometer (Switzerland). 20 mg purified and freeze-dried levan was dissolved in D₂O [24], dissolving and lyophilizing repeatedly to realize the exchange of H and D. The prepared sample was sealed in an NMR tube. All the experiments were performed at room temperature and all the data were analyzed with MestRenova software.

3. Results And Discussion

3.1 Purification of the recombinant levansucrase

As shown in Fig. 1, the purified enzyme had a single band clearly visible in SDS-PAGE (between 80kDa and 58kDa), indicating that no impurities were introduced in the purification process. GEM particle purification is a promising method, which can save purification time, simplify operation and reduce activity loss compared with traditional purification methods [21].

3.2 Effect of temperature, pH, metal ions on the levan formation activity of the recombinant levansucrase

The levan biosynthesis ability of the recombinant enzyme was evaluated at various temperatures, ranging from 20 °C to 60 °C. As shown in Fig. 2A, the optimum activity temperature of the recombinase was 40 °C, and the activity of the recombinase was severely inhibited when temperatures below 30°C and above 55°C. The levansucrase from *Bacillus methylotrophicus* SK 21.002 showed the highest levan production at around 37 °C [25]. Lower optimum temperature for levan formation of 15 °C was reported for levansucrase from *Halomonas smyrnensis* AAD6^T [26].

The effect of pH on the levan biosynthesis ability of the recombinant enzyme was studied at different pH values (4.6–5.6). The highest levan formation activity of enzyme was obtained at pH 5.2. As shown in the Fig. 2B, the activity was rather sensitive to pH which was only high at pH 5.2 but decreased sharply when pH below 5.0 or above pH 5.4. The optimum pH for levan synthesis was lower than that of levansucrase from *Leuconostoc mesenteroides* B-512 FMC [27] and levansucrase from *Brenneria goodwinii* [28], and their optimum pH values were 6.2 and 6.0, respectively. The optimal pH for most levansucrases is between 5.0 and 6.5 [29].

The effects of different concentrations and different kinds of metal ions on the levan formation activity of levansucrase were determined at pH 5.2 and 40 °C (Fig. 2C). 50 mM Ca²⁺ and K⁺ increased the activity to around 130% of the initial relative activity. However, metal ions such as Cu²⁺, Fe³⁺ and Zn²⁺ had an obvious inhibitory effect on the enzyme activity indicating that the recombinase was sensitive to the presence of Cu²⁺, Fe³⁺ and Zn²⁺. Levansucrase from *Bacillus methylotrophicus* SK 21.002 was tested for effects on metal ions on levan biosynthesis. 20 mM Mg²⁺ increased the enzyme activity to 115% of the initial relative activity, while Cu²⁺, Fe²⁺, and Zn²⁺ had a strong inhibitory effect on the enzyme activity [25].

Hg^{2+} and Ag^+ decreased the activity of levansucrase from *Leuconostoc Mesenteroides* B-512 FMC by 92% and 86%, respectively, while Zn^{2+} , Fe^{2+} and Cu^{2+} slightly inhibited the activity of levansucrase [27].

Levan biosynthesis was carried out from 10% (w/v) sucrose at pH 5.2 and 40 °C, using the recombinant levansucrase of 6.45 U/g sucrose. The highest production reached to 30.6 g/L after 2 h, which was higher than 15 g/L for *Erwinia herbicola* and [30] and lower than 36 g/L for *B. polymyxa* (NRRL B-18475) [31].

3.3 Enzyme kinetics

Different concentrations of sucrose solution were prepared with sodium acetate-acetate buffer. The reaction rate of levansucrase with different concentrations of sucrose solution was determined according to the method described in 2.5. According to the regression equation (Fig. 3), the Michaelis constant of levansucrase to sucrose was 25.63 mM. The K_m value of the enzyme in this study was similar to the K_m value (24mM) of the levansucrase from *Leuconostoc mesenteroides* NTM048 [11]. Moreover, the K_m value was much lower than that of levansucrase from *Halomonas smyrnensis* AAD6^T (104.79 ± 4.17 mM) [26]. Therefore, the affinity of levansucrase from different microbial sources to sucrose is very different.

3.4 Monosaccharide Composition Analysis

Monosaccharide composition analysis usually requires hydrolysis of polysaccharides or oligosaccharides with appropriate acids before derivatization for gas chromatography (GC) and high-performance liquid chromatography (HPLC) analysis, or high-performance ion chromatography (HPIC) analysis without derivatization. The resulting chromatogram is shown in Fig. 4. By comparing the retention time of sample monosaccharides with that of standard monosaccharides, it was determined that the sample polysaccharide was composed of fructose, glucose and galactose, which accounted for 81.6%, 16.6% and 1.9%, respectively. The monosaccharide composition of polysaccharides is related to many factors, such as the hydrolysis temperature of the sample can affect the extraction of ketose. When the temperature was 30–70°C, the free fructose was relatively stable; when the temperature rose to 120°C, the free fructose would rapidly degrade to 80% [32].

3.5 Molecular weight and purity of levan

Gel permeation chromatography is the most commonly used method to detect the purity and molecular weight of polysaccharides. The molecular weight of levan was obtained by comparing the retention time of levan with the standard substance with different molecular weights. The retention time of levan was 16.667 min and it has a single elution peak in gel permeation chromatography (Fig. 5), indicating that the polymer is a homogeneous component. Based on the linear regression curve of PEG standards, the average molecular weight of levan was 1.56×10^6 Da by calculation. In general, the molecular weight of polysaccharides is related to many factors, including strain type, fermentation conditions, medium composition, and extraction method. Malang et al [33] have shown levans synthesized by raffinose as carbon source in *W. confusa* E5/2 – 1 have higher molecular weight than levans synthesized from

sucrose. Levan produced by *Bacillus subtilis* was reported to have two levan distributions: a high molecular weight levan (2.3×10^6 Da) and a low molecular weight levan (7.2×10^3 Da) [34]. The molecular weight of levan in this study is between the two. Levan with different molecular weights has different applications in medicine, cosmetics and food. For example, levan with low Mw produced from *Z. Mobilis* had a stronger antibacterial inhibition in vitro, while levan with high Mw produced from *Bacillus subtilis* NRC1aza had the strongest DPPH free radical scavenging activity [35].

3.6 SEM analysis

A scanning electron microscopy analysis was performed to observe the microstructure and surface morphology of the levan, which can help in understanding the physical properties of the levan. The surface morphology micrographs of levan at 400 \times , 1000 \times , 2000 \times were shown in Fig. 6. As observed by SEM images, levan in this study had a highly branched and porous structure. It was supposed that levan with a highly branched and porous structure was conducive to the formation of hydrated polymers and was most likely to be used in foods and cosmetics industries as texturing, thickening, stabilizing, and water-binding agent [36–38]. Besides, SEM images indicated that levan had a sheet-like smooth and glossy surface, which had the potential to prepare plasticized film [39]. The part microstructure of the levan in this study was similar to the microstructure of glucan produced by *Leuconostoc pseudomesenteroides* XG5, which had a smooth and glittering surface and high branched structure [38], but there were a little differences between levan from *Bacillus mojavensis* and *Brenneria* sp. EnID312 exhibited uniform porous network [7, 40].

3.7 AFM analysis

AFM is a useful tool for characterizing polymer morphology with high resolution and simple operation, which is developed on the basis of SEM. The topographical AFM images of levan exhibited many ellipsoidal or spheroidal particles and spike-like lumps (Fig. 7), which indicated that polysaccharides had a strong affinity with water molecules [40–42]. The maximum peak height of rounded lumps was 55.7 nm, the average roughness was 3.41 nm and the mean roughness was 1.48 nm. The maximum height of levan was much higher than the height of a single polysaccharide chain(0.1-1nm) suggesting that the tightly packed molecular structure formed in AFM images may be caused by the intermolecular and intramolecular aggregation of levan [36]. A similar result was reported for the EPS polymer from *Lactobacillus sakei* L3 [43] but different from *Lactobacillus reuteri* E81 glucan which had the tangled networks [44] and Mesona blumes gum EPS polymer which had an irregular shape like the worm [45].

3.8 FT-IR analysis

Fourier-transformed infrared spectroscopy is used to determine the glycoside bond configuration and the functional group on the sugar chain by using the relative vibrations within the molecule and molecular rotation information to analyze the structure of polysaccharides. Figure 8 showed the FT-IR spectrum of purified levan. The wide and strong peak at 3304 cm^{-1} was caused by the stretching vibration of O-H [25,

46], indicating the existence of intermolecular hydrogen bonding. The weak peaks at 2931 cm^{-1} and 2887 cm^{-1} were the results of C-H stretching vibration and bending vibration respectively [47]. The strong absorption peak at 1644 cm^{-1} was caused by the O-H bending vibration, which might be caused by the presence of water in the sample [48]. The absorption peaks at 1122 cm^{-1} and 1009 cm^{-1} were caused by C-O-C stretching vibration [49], which were the characteristic peaks of carbohydrates. The absorption peaks at 923 cm^{-1} and 809 cm^{-1} represented symmetric stretching vibration of furanose and D-type C-H bending vibration of furanose respectively, which were typical signal peaks of furanose [14, 50]. Thus it was proved that a furan ring was contained in the polysaccharide structure. Preliminary analysis showed that the polysaccharide was composed of D-furanose.

3.9 NMR analysis

Further analysis of the structure of purified polysaccharides was obtained by ^1H and ^{13}C NMR spectra. The ^{13}C NMR spectra had several signals in the anomeric carbon signal region (95-110ppm). The peak at 96.31 ppm may be a signal for α -glucose C1 [51]. Two peaks at 104.19 and 103.65 ppm were derived from β -fructose C2 signal [52]. Major signals in the ring carbon signal region (50-85ppm) at around 59.88, 76.28, 75.18, 80.27 and 63.36 ppm were attributed to the fructose groups C1, C3, C4, C5 and C6, respectively. Among them, signal at the 63.36 ppm (C6) confirmed the presence of the fructose β -(2,6)-linkage [53]. These carbon chemical shift of levan produced by levansucrase reported in other literatures are shown in the table below, which are similar to the six signals in this paper.

According to the ^1H NMR spectra, the signal at 4.70 ppm was due to D_2O . The signals of 5.33 and 5.15 ppm in the anomeric proton region can be attributed to the characteristic signal of α -glucose H1 [39]. Seven major proton signals were observed at 4.10 ppm (H3), 4.01 ppm (H4), 3.87 ppm (H5), 3.82 ppm (H6a), 3.68 ppm (H1a), 3.60 ppm (H1b), and 3.48 ppm (H6b), respectively. All of them were in the ring proton region (3.4–4.2 ppm) indicating that the presence of fructose in the polysaccharide [50]. The ratio of peak areas was approximately 1:1:1:1:1:1 which meant the same amount of every kind of H atom. No signal of galactose residue was found in the spectrum, which may be due to the low content of galactose residues, resulting in too little signal accumulation. All the information indicated that the polysaccharide synthesized by levansucrase was β -(2,6) levan.

4. Conclusion

In this study, levan was synthesized in vitro with recombinant levansucrase from *Bacillus subtilis* ZW019 strain. The yield of levan reached 30.6 g/L. The synthesized levan has a molecular weight of 1.56×10^6 Da, and its structure is β -(2,6) fructose, containing furan ring. The results indicated that the genetically engineered *E. coli* strain could express levansucrase efficiently, and the recombinant levansucrase could be easily purified in one step. Moreover, the recombinant levansucrase showed high catalytic activity in vitro, and the composition and structure of the synthesized levan were highly similar to that of natural

levan. In the future, we will further study the biological function of the levan synthesized by enzymatic method, and then explore the application way of the levan.

Declarations

Author Contributions

Jingyue Wang: Conceptualization, Methodology, Investigation, Writing - Original Draft.

Xinan Xu: Conceptualization, Methodology, Investigation, Writing - Original Draft.

Fangkun Zhao: Investigation, Writing - Review & Editing, Formal analysis.

Nan Yin: Investigation, Formal analysis.

Zhijiang Zhou: Supervision, Software.

Ye Han*: Resources, Supervision, Project administration, Funding acquisition.

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Conflict of interest

The authors declare no conflict of interest and unanimously approve publication of the manuscript.

Data Availability Statement

All data included in this study are available upon request by contact with the corresponding author.

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Table

Table 1 ^{13}C chemical shifts of levans produced by different levansucrases

Microorganisms	C1	C2	C3	C4	C5	C6	References
<i>Lactobacillus reuteri</i> LTH5448	60.93	104.55	77.25	75.90	80.66	63.81	[14]
<i>Leuconostoc mesenteroides</i> NTM048	59.91	104.24	76.30	75.22	80.33	63.44	[11]
<i>Bacillus licheniformis</i> ANT 179	62.58	106.80	78.96	77.83	82.89	65.99	[3]
<i>Bacillus methylotrophicus</i> SK 21.002	61.20	104.66	77.51	76.10	80.77	63.94	[25]
<i>Bacillus subtilis</i> ZW019	59.88	104.19	76.28	75.18	80.27	63.36	this study

Figures

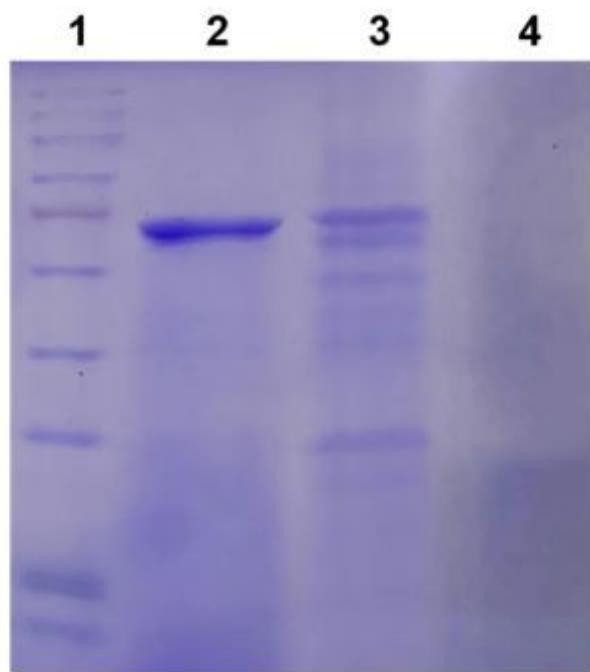


Figure 1

The levansucrase was purified by GEM. Lanes: 1, Molecular mass standards; 2, Purified intracellular recombinant levansucrase; 3, The effluent after purification 4, GEM particles.

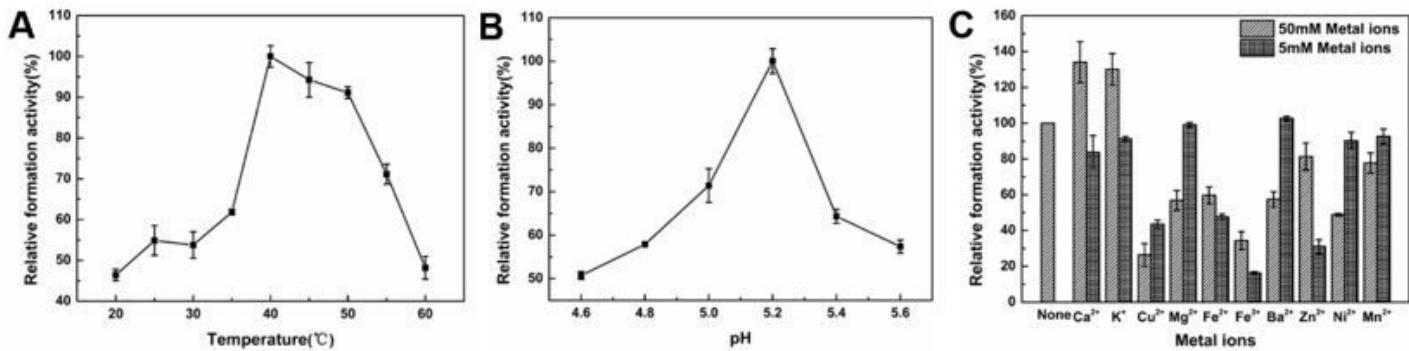


Figure 2

Effect of temperature (A), pH (B), metal ions (C) on the levan formation activity of recombinant levansucrase.

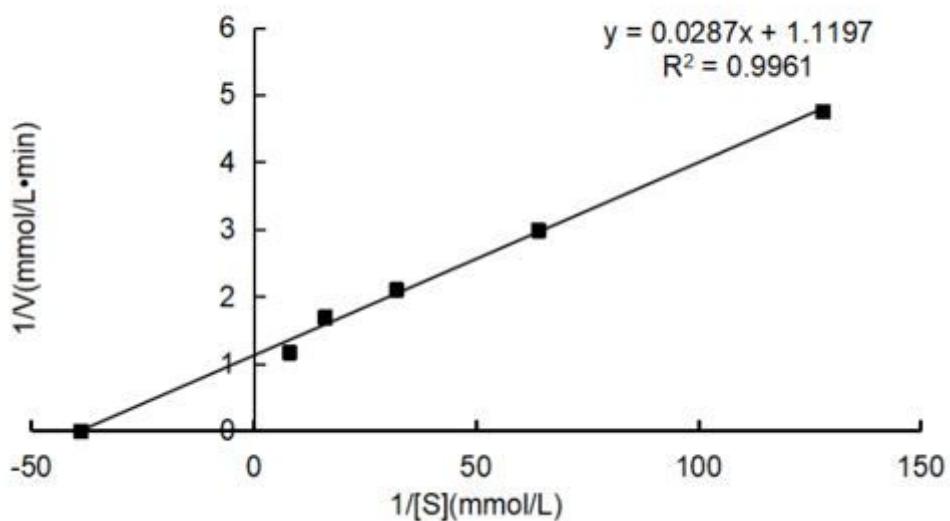


Figure 3

Michaelis–Menten kinetic parameters

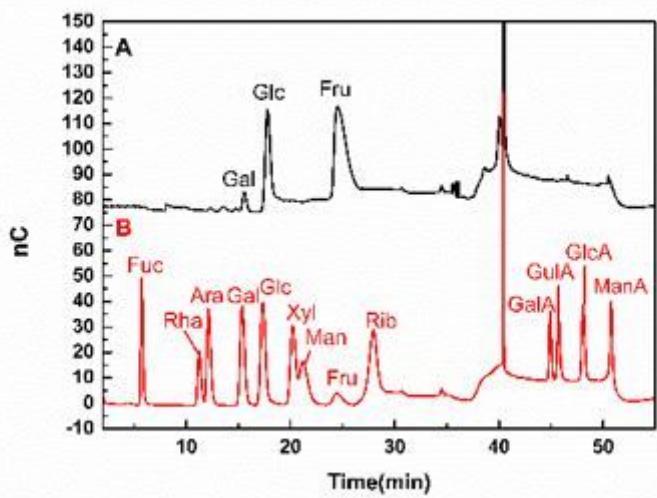


Figure 4

Sugar analysis spectrum of purified levan.

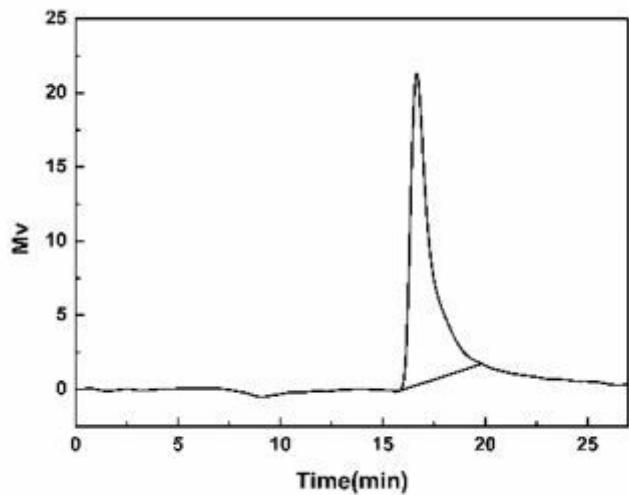


Figure 5

Gel permeation chromatography of purified levan.

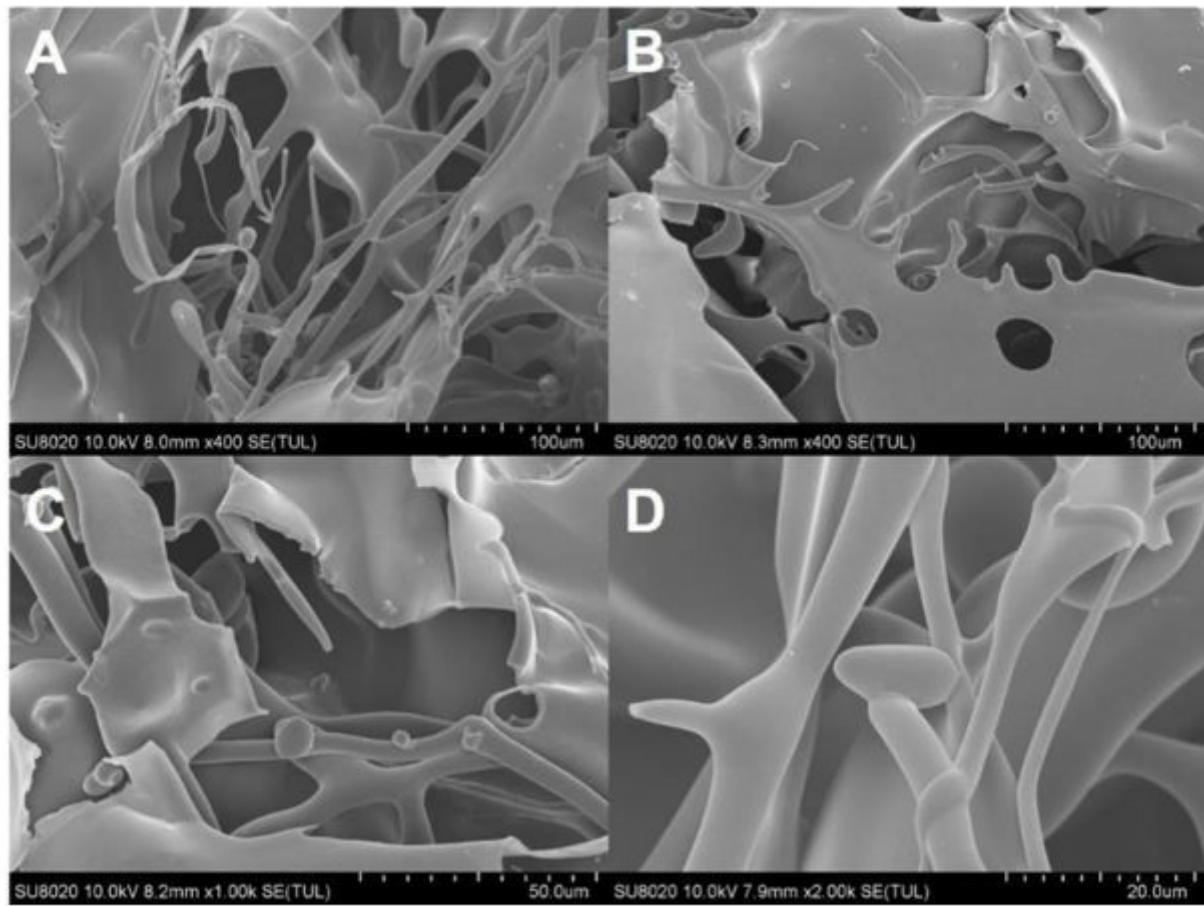


Figure 6

SEM images of purified levan at 400× (A,B), 1000× (C), 2000× (D) magnification.

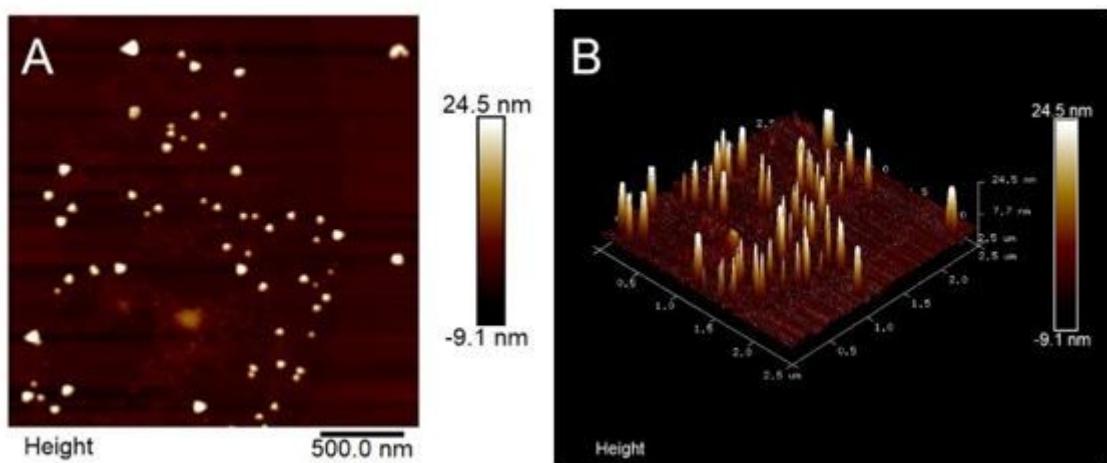


Figure 7

AFM images of purified levan (A) planar and (B) cubic.

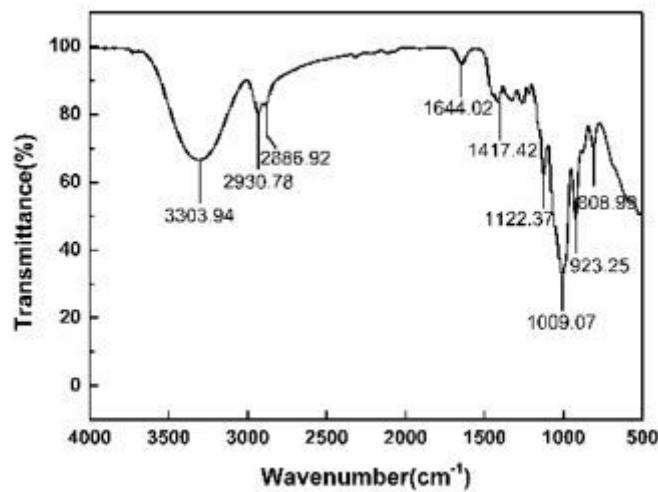


Figure 8

FT-IR spectra of purified levan.

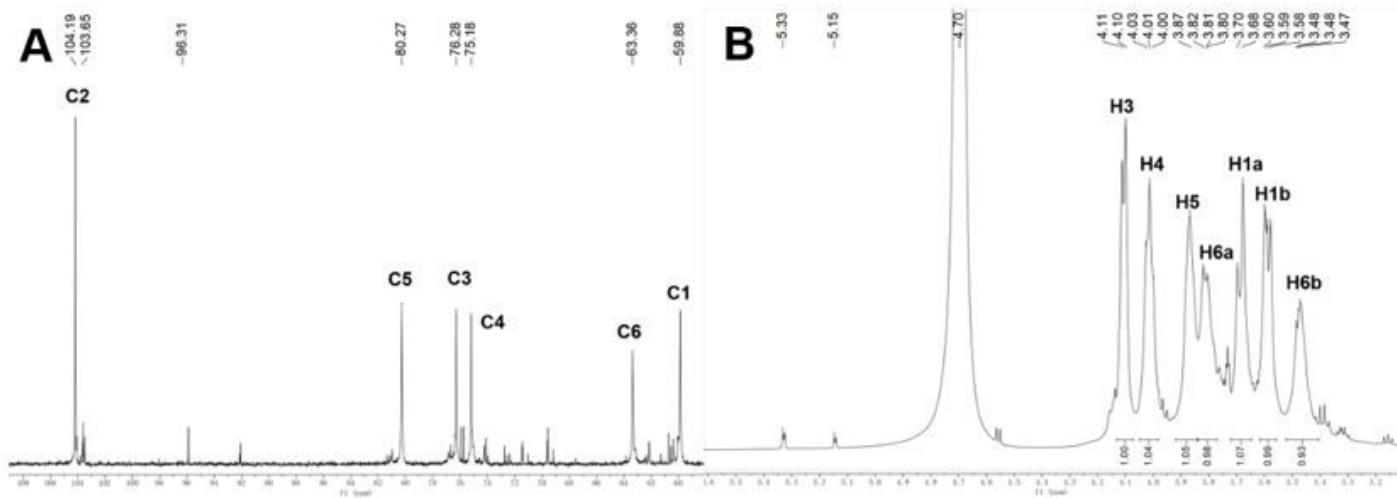


Figure 9

(A) 1D ^{13}C and (B) ^1H NMR spectra of purified levan.

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