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Biosynthesis of levan by levansucrase from *Bacillus methylotrophicus* SK 21.002

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ABSTRACT

A new strain SK 21.002 which could produce intracellular levansucrase was isolated from beet sugar growing soil. It was identified as *Bacillus methylotrophicus* based on the sequence analysis of the 16S rRNA and the morphological characteristic. The structure of levan biosynthesized from the levansucrase was determined to be connected through β-(2 → 6) linkages with fructofuranosyl residues. The molecular weight of the product was between 4 and 5 kDa. The optimal conditions to produce levan using the levansucrase were studied. The presence of Mg^{2+} could increase the yield of levan. The yield of levan reached about 100 g/L with the enzyme under the optimum conditions.

1. Introduction

Biopolymers are superior to petrochemical-derived polymers in several aspects that embrace biocompatibility, biodegradability, and both environmental and human compatibility (Poli et al., 2009). Levan is a kind of natural homopolysaccharides. It is consisted of a sucrose molecule which is elongated by a chain of fructosyl units connected through β-(2 → 6) linkages (Han, 1990). Potential industrial applications of levan have been proposed as an emulsifier, formulation aid, stabilizer and thickener, surface-finishing agent, encapsulating agent, and carrier for flavor and fragrances (Shih, Yu, Shieh, & Hsieh, 2005). In addition, levan is promising in medicine as plasma substitute, drug activity prolongator and antihyperlipidemic agent (Yamamoto et al., 1999).

Levan is naturally in plants, such as *Triticum aestivum*, *Cocksfoot*, *Pachysandra terminalis* (Ende et al., 2011; Feingold & Gehatia, 1957; Praznik, Spies, & Hofinger, 1992). It exists in the growth process of plant cells to store water and control active ingredients in the fixed area, which could protect cells. Meanwhile, levan can play a positive effect on stress tolerance of plants (Pavis et al., 2001). According to this theory, levan has remarkable cell-proliferating, skin moisturizing, and skin irritation-alleviating effects as a blending component in cosmetics (Abdel-Fattah, Gamal-Eldeen, Helmy, & Esawy, 2012). However, the content of levan in plants is very low (Ende et al., 2011; Feingold & Gehatia, 1957; Praznik et al., 1992). Thus, it is impossible to obtain sufficient quantities of levan from natural sources to satisfy the applications of food, medicine and cosmetic industries.

It is a good way to produce levan with microorganism. As early as 1902, Greig-Smith and Steel had found one microorganism that could produce levan from the secretion of *Eucalyptus stuarina* (Greig-Smith & Steel, 1902; Kopeloff, Kopeloff, & Welcome, 1920). Since then, new sources of levan are being sought. So far, there are some researches and reports about microorganisms that can produce levan. They are mainly bacteria, including *Acetobacter xylinum* (Tajima et al., 1998), *Bacillus subtilis* (Shih, Chen, & Wu, 2010), *Microbacterium laevaniformans* (Bae, Oh, Lee, Yoo, & Lee, 2008), *Bacillus amyloliquefaciens* (Tian, Inthanavong, & Karboune, 2011), *Bacillus polymyxa* (Han & Watson, 1992), *Zymomonas mobilis* (Chiang, Wang, & Chen, 2009), *Lactobacillus sanfranciscensis* (Tieking, Ehrmann, Vogel, & Gänzle, 2004), *Leuconostoc mesenteroides* (Morales-arrieta, Rodríguez, Segovia, & López-Mugía, 2006) and *Pseudomonas syringae* (Visnapuu, Mardo, Mosoarca, Zamfir, & Vigants, 2011). However, few of them have high yield of levan, which limits the large-scale industrialization and application of levan. Beyond that, the complex process of purifying levan also limits the production and application. Once the limitations are solved, the market for levan will gradually increase in many fields.

In this study, a new levan producing strain *Bacillus methylotrophicus* (*B. methylotrophicus*) was isolated and characterized. The structure of the biosynthetic product was determined to be levan. The conditions of levan biosynthesis from sucrose with the enzyme from the strain fermentation were investigated. At the end,

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a more efficient method to produce levan was developed. To our knowledge, it is the first report that *B. methylotrophicus* has the levansucrase and levan producing ability.

2. Materials and methods

2.1. Materials

Several soil samples were collected from beet and sugarcane gardens in Haerbin and Nanning, China, respectively. Standards dextran T-2000, dextran T-580, dextran T-70, dextran T-10, dextran T-5 and maltose were purchased from Sigma (St. Louis, MO, USA). Other chemicals were of analytical grade and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Strain isolation and identification

The soil samples (1 g) were respectively suspended in sterilized water and spread on agar plates containing (g/L): sucrose 20, yeast extracts 10, peptone 5, K_2HPO_4 4.5, and agar 20 (pH 7.5). The plates were incubated at 30 °C in constant temperature incubator for 24 h. Growing colonies were picked up, and then were suspended in the liquid medium containing (g/L): sucrose 20, yeast extracts 10, peptone 5, K_2HPO_4 4.5, pH 7.5. The media was autoclaved at 121 °C for 20 min and then cooled to room temperature before inoculation. The incubation was carried out in a rotary shaker (180 rpm) at 30 °C for 24 h. Then the cells were collected by centrifugation at 8000 × *g* for 15 min at 4 °C, washed twice with 20 mM sterile sodium phosphate buffer (pH 6.0), and resuspended in sodium phosphate buffer (pH 6.0) by adding 10 mL buffer to 1 g wet cells. The cells were disrupted by ultrasonication (190 W, pulse on, 1 s; pulse off, 2 s) for 15 min. The resulting suspension was shaken at 4 °C for 1 h to liberate the membrane-associated levansucrase. The unbroken cells and cellular debris were removed by centrifugation (10,000 × *g*, 10 min) at 4 °C. The supernatant was collected as crude enzyme to produce levan. The methods of levan biosynthesis and the ability assay were described below. The strain with high levan productivity was selected for further research and then identified.

The 16S rRNA gene was amplified with bacterial universal primers 27F (5′-AG AGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTA CCTTGTTACG ACT T-3′), which were used for sequencing. Homology researches were performed against the sequences of database using the BLAST program (NCBI, Bethesda, MD, USA).

In order to observe the cellular morphology, the cells sample was prepared in the following steps. Firstly, the cells were fixed for 2 h by glutaraldehyde (3%) at 4 °C. And the cells were collected by centrifugation (1500 × *g*, 3 min) at 4 °C, washed four times with 0.1 M sterile sodium phosphate buffer. Then the cells were spotted on the copper grid of TEM (Ø 3 mm, 200 meshes) and were naturally dried in the air. Next the cells were stained by uranyl acetate (3%) and were naturally dried again in the air. At last cellular morphology was observed by transmission electron microscope (TEM, Hitachi H-7650, Japan).

2.3. Isolation and purification of levan

At the end of the enzymatic synthesis reaction (the reaction method illustrated in Section 2.7), the supernatant was treated with Sevag reagent (*n*-butanol: chloroform = 1:4, v/v) to remove any proteins (Sevag, Lackman, & Smolens, 1938). This step was repeated until there was no absorption under 280 nm, as measured on a Cary 50 UV–Vis spectrophotometer (Varian Inc., USA). After that, the supernatant solution without protein was precipitated using 3 volumes of anhydrous ethanol and kept at 4 °C with stirring overnight. The resulting precipitate was collected by centrifugation at 10,000 × *g* for 10 min at 4 °C. The precipitate was redissolved in deionized water followed by centrifugation to remove any insoluble materials, and then reprecipitated with 4 volumes of anhydrous ethanol at 4 °C overnight. After centrifugation at 10,000 × *g* for 10 min at 4 °C, levan was obtained by the supernatant lyophilized.

2.4. Levan structural characterization and identification methods

2.4.1. Relative molecular measurement

The molecular weights of the purified levan (redissolved with deionized water to 2 mg/mL) were determined by high performance gel filtration chromatography (HPGFC), which was performed on a Waters 600 HPLC system with an Ultrahydrogel™ Linear (300 mm × 7.8 mm id × 2) gel filtration column eluted with 0.1 M $NaNO_3$ at a flow fate of 0.9 mL/min. Standard dextrans T-2000 (2000 kDa), T-580 (580 kDa), T-70 (70 kDa), T-10 (10 kDa), T-5 (2.9 kDa) and maltose (0.342 kDa) were also applied to the same system. The molecular weight distributions of the samples were determined by comparison with the retention time of standards under the same conditions.

2.4.2. Sugar analysis of the hydrolyzed product

For sugar analysis, production samples (2–3 mg) were hydrolyzed with 1 M sulfuric acid at 105 °C for 4 h. The hydrolytic reaction was stopped by adding excess $BaCO_3$ powder to the hydrolytic solution. The sugar compositions of the sample were analyzed by HPLC as followed.

2.4.3. ^1H and ^{13}C NMR spectra

For NMR analysis, ^1H and ^{13}C NMR spectra of levan were performed on a Brucker AVANCE III-400 MHz spectrometer at 70 °C. ^1H experiment was recorded at the operating frequency of 400.13 MHz and was recorded 16 times. The scanning width was 8223 Hz, while the relaxation time was 1 s. ^{13}C NMR experiments were obtained at the operating frequency of 100.57 MHz and was recorded 1402 times. The scanning width was 24,038 Hz, while the relaxation time was 2 s.

2.4.4. Fourier transform-infrared spectroscopy

The basic compositions of levan were determined by Fourier Transform Infrared spectrometer (FT-IR). Potassium bromide tablet was adopted to pre-treat the samples. Sample was mixed with KBr in a ratio of 1:100, ground thoroughly and then pressed into a 1 mm pellet. The spectra were recorded in the absorbance mode from 4000 to 400 cm^{-1} with a 4 cm^{-1} resolution on a Thermo Nicolet NEXUS 470 FT-IR (Thermo Fisher Scientific, USA).

2.5. Enzyme activity assay

The enzyme reaction was assayed by mixing 0.1 mL enzyme solution with 0.4 mL 20 mM sodium phosphate buffer (pH 6.0) and 0.5 mL of 20% sucrose (w/v) in 20 mM sodium phosphate buffer (pH 6.0) preheated at 40 °C, and immediately incubation of the reaction mixture at 40 °C for 20 min. The reaction was stopped in boiling water for 10 min. One unit of total levansucrase activity is defined as the amount of the biocatalyst to produce 1 μmol of glucose per minute.

2.6. HPLC analysis of sugars

The bio-productions of glucose, fructose, oligosaccharides, and levan were determined by HPLC with a differential refractive index detector and Sugar-Pak™ I column packed with a microparticulate cation-exchange gel in calcium form (6.5 mm × 300 mm). The

column temperature was 85 °C. Deionized water was used as a mobile phase at 0.4 mL/min.

2.7. Optimization of levan biosynthesis

Enzymatic synthesis reactions were carried out by using a 10 mL reaction mixture containing 250 g/L sucrose prepared with 20 mM sodium phosphate buffer (pH 6.0) and 6 U/g levansucrase and incubated at 40 °C for 24 h in a shaking water bath. The reactions were terminated in boiling water for 10 min. Then the supernatant was obtained by centrifugation at 10,000 × *g* for 10 min (4 °C). The production of levan was determined by HPLC as above.

2.7.1. Effect of substrate concentration on the levan biosynthesis

Sucrose concentrations from 100 to 400 g/L were used to optimize the substrate concentration. Others were the same as the initial reaction conditions.

2.7.2. Effect of enzyme dosage on the levan biosynthesis

The reaction mixtures were prepared with the optimal sucrose concentration and different enzyme dosages of 1, 2, 4, 6, 9 and 12 U/g sucrose. Others were the same as the initial reaction conditions.

2.7.3. Effect of pH value on the levan biosynthesis

The effect of pH values on levan production was carried out with the pH ranging from 5.0 to 8.0, while the sucrose concentration and enzyme dosage were in the optimal conditions, respectively. Afterward the reaction mixtures were incubated at 40 °C for 24 h.

2.7.4. Effects of metal ions on the levan biosynthesis

In order to investigate the effects of metal ions on levan biosynthesis, the reaction mixtures containing 20 mM Ba^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, and K^{+} were prepared, respectively. The sucrose concentration, enzyme dosage and pH value were kept at the optimal conditions. Then the reaction mixtures were incubated at 40 °C for 24 h.

2.7.5. Effect of temperature on the levan biosynthesis

To determine the optimum temperature for levan production, the reaction mixtures under the optimal concentrations were incubated at 25 °C, 30 °C, 37 °C, 40 °C, 45 °C, 50 °C, and 55 °C for 24 h, respectively.

2.8. Levan biosynthesis

Production of levan from sucrose was studied with the levansucrase under the optimum conditions. Samples were taken at appropriate time intervals and analyzed for sugar content HPLC.

2.9. Statistical analysis

The levan concentration was expressed as mean ± standard deviation of three different experiments. Statistical calculation was performed using the GraphPad Prism® package (GraphPad Software Inc., San Diego, CA, USA).

3. Results and discussion

3.1. Screening and identification of strain with levansucrase

One strain named SK 21.002 with highly producing intracellular levansucrase was screened from beet soil. The characteristics of the isolate were Gram-positive, endospore-forming, and rod-shape. The 16S rRNA gene sequence of the strain SK 21.002 was obtained by using general primers in the PCR. And the 16S rRNA gene of

a

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TTCGGCGGCTGGCTCCTAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCT
CGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCAT
GCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGC
GATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGC
CCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGAT
GATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAG
TGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACT
TAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACT
CTGCCCCCGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCT
GGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCG
GGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGA
GTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAG
CACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCAC
GCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGT
GTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCT
TCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCT
TTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTAACGCCCAATAATTCCGG
ACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCAAGTAGTTAGCCGTGG
CTTTCTGGTTAGGTACCGTCAAGGTGCCGCCCTATTTGAACGGCACTTGTTCTT
CCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGC
TCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGA
GTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTAC
GCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGT
CCATCTGTAAGTGGTAGCCGAAGCCACCTTTTATGTCTGAACCATGCGGTTCAA
ACAACCATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCA
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ATCTGTCCGCTC
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b

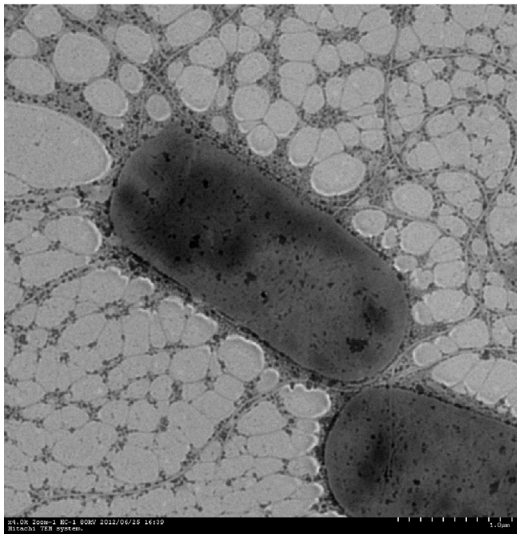

Fig. 1. Identification of the strain SK21.001. (a) The 16S rRNA sequence of the isolated strain, (b) the surface morphology of SK21.001 by TEM and (c) growth of *Bacillus methylotrophicus* SK 21.002 bacteria on agar plate with sucrose.

strain SK21.002 was submitted to Genebank (accession number KC978718). Its 16S rRNA sequence (Fig. 1a) showed 99.85% similarity with *B. methylotrophicus* 16S rRNA gene (EU194897) from the BLAST result. The strain was deposited in the China Center for Type Culture Collection (CCTCC) with the accession number M2011476.

The internal morphology (Fig. 1b) of the strain was observed by TEM. When the culture was grown on agar with sucrose, the bacterial colonies had a mucoid appearance, which indicated the production of polysaccharide from sucrose. When *B. methylotrophicus* SK 21.002 grew on a sucrose-containing liquid medium, the levansucrase was induced and levan was produced, and which was confirmed by the following structure identification.

Accordingly, the strain SK21.002 with levansucrase was confirmed and classified as *B. methylotrophicus* species and named as *B. methylotrophicus* SK21.002.

3.2. Purification, structure identification and characterization of levan

The biosynthesis production levan with the levansucrase from *B. methylotrophicus* SK 21.002 was purified to be homogenesis according to the purification method as illustrated in Section 2.

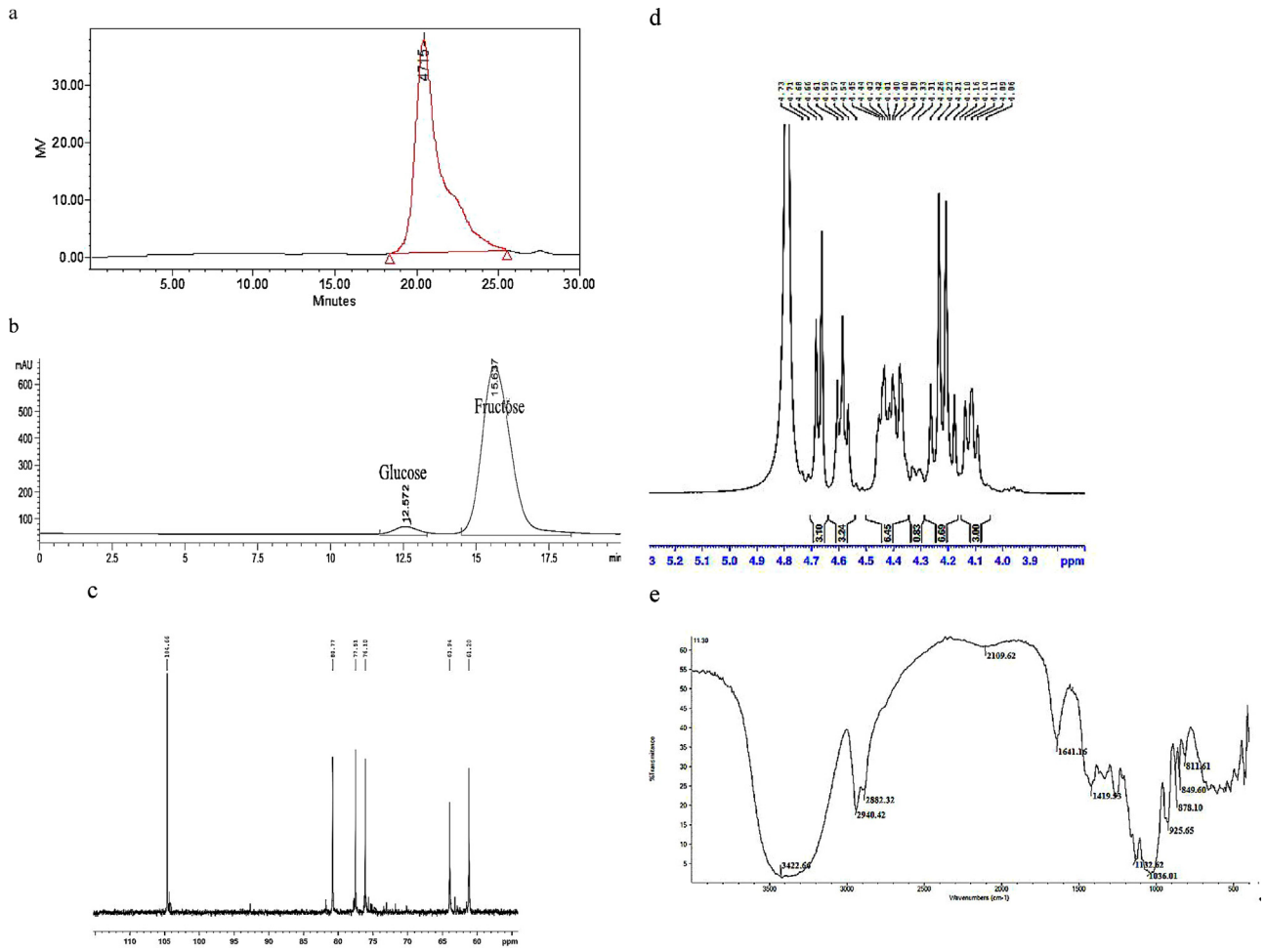

Fig. 2. Structure identification of levan. (a) Relative molecular spectrum, (b) sugar analysis spectrum, (c) ^{13}C NMR spectrum, (d) ^{1}H NMR and (e) Fourier transform-infrared spectroscopy spectrum.

The result shown in Fig. 2a indicated that the molecular weight of the levan was 3816 Da. The levansucrase presented in the current study produced levan with low molecular between 4 and 5 kDa, which was different from levansucrases from other bacteria (Mattoon, Holmlund, Schepartz, Vavra, & Johnson, 1955; Viikari & Gisler, 1985) producing levan with high molecular.

The purified levan was hydrolyzed by 2 M sulfuric acid at 105 °C for 4 h and then analyzed by HPLC. The sugar analysis (Fig. 2b) of the hydrolysate indicated that the product consisted of glucose and fructose.

Further, the ^{13}C NMR spectrum (Fig. 2c) of the product showed six broad resonance signals at 104.66 (C2), 80.77 (C5), 77.51 (C3), 76.10 (C4), 63.94 (C6), 61.20 (C1) ppm. The carbon chemical shifts were ascribed to β-configurated fructofuranose units by comparison with the carbon chemical shifts of the standard methyl glycoside (Bock & Pedersen, 1983). These values confirmed the identification of the product as being a levan since they were almost identical to the literature values of levan ^{13}C NMR spectrum (Shimanmura, Tsuboi, Nagase, & Ito, 1987; Simms, Boyko, & Edwards, 1990). The ^{1}H NMR spectrum (Fig. 2d) of the product did not exhibit any peaks in anomeric region.

The FT-IR spectrum in Fig. 2e showed the characteristic absorption of levan. The stretching vibration of O—H falls within the wave number range of 3600–3200 cm^{-1} (Silverstein, Webster, & Kiemle, 2005). The broad and pure peak at 3422.66 cm^{-1} indicated intermolecular hydrogen bonding. The bands within the region of 3000–2800 cm^{-1} were due to the C—H stretching vibration. The peak at 1641.16 cm^{-1} was the stretching vibration of C—O. Several sharp peaks between 1000 cm^{-1} and 800 cm^{-1} were the typical peaks of carbohydrates. The FT-IR spectrum of levan featured a strong absorption at 2090 cm^{-1}, indicating the presence of β-type glycosidic linkages.

All these data indicated the levan structure of the biosynthesis product from sucrose by *B. methylotrophicus* SK 21.002 levansucrase.

3.3. Optimization of levan biosynthesis

The biosynthesis conditions (including substrate sucrose concentration, levansucrase dosage, reaction pH, temperature, and time) were optimized by step-by-step method to achieve levan by high efficiency, high quality and high yield.

3.3.1. Effects of sucrose concentration on levan biosynthesis

The effect of sucrose concentration on levan production was investigated (Fig. 3a). The result revealed that the sucrose concentration was directly proportional to the production of levan. However, the increasing rate of levan production reduced and tended to be steady when the sucrose concentration was above 300 g/L. This is due to the increasing of osmotic pressure and the viscosity of the reaction system with the substrate concentration increase, which leads to the enzyme activity inhibited and the speed of molecular moving slowed. Therefore, sucrose of 300 g/L was the optimal substrate concentration based on the high

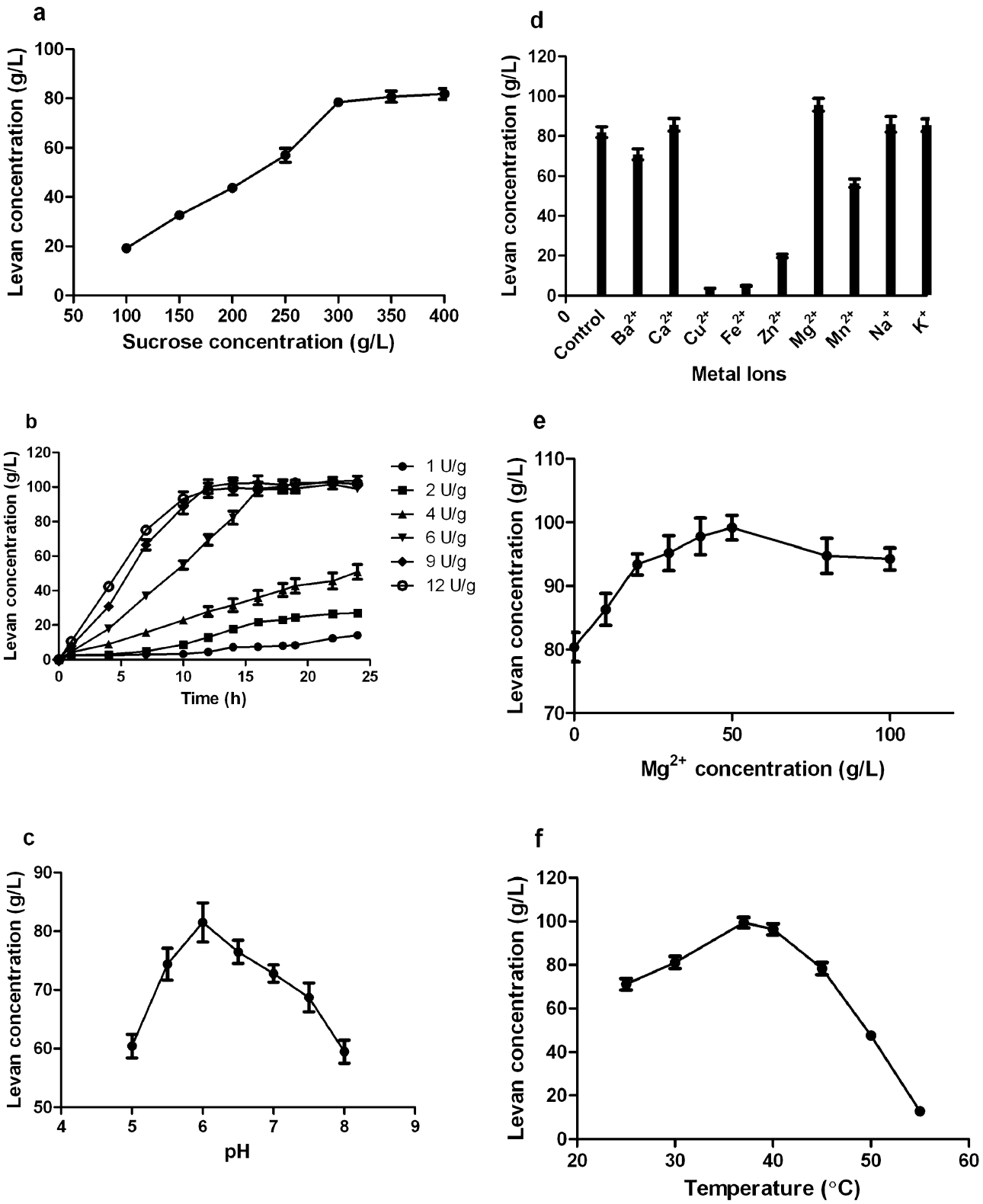

Fig. 3. Effect of biosynthesis conditions on the production of levan. (a) Effect of sucrose concentration, (b) effect of enzyme concentration, (c) effect of pH, (d) effect of metal ions, (e) effect of Mg^{2+} concentration and (f) effect of temperature.

efficiency. The result indicated that at higher sucrose concentration the transfructosylation reaction was performed better, which made the *B. methylotrophicus* SK 21.002 levansucrase different from the reported levansucrase. Hettwer, Gross, and Rudolph (1995) reported that at higher sucrose concentrations (more than 10%), the hydrolysis reaction was over the transfructosylation reaction. Thus, the levansucrase produced by *B. methylotrophicus* SK 21.002 was a promising candidate for industrial applications.

3.3.2. *Effects of enzyme dosage on levan biosynthesis*

With 300 g/L sucrose and other conditions fixed, enzymatic reaction rate is proportional to the enzyme dosage. The effect of

enzyme dosage on levan production was investigated (Fig. 3b). The result showed that when the enzyme dosage was lower than 4 U/g sucrose, the reaction period to reach balance was very long and over 24 h, while it only took 12 h with the enzyme dosage at 9 U/g sucrose or 12 U/g sucrose. And when the enzyme dosage was 6 U/g sucrose, it took 16 h to reach balance and the levan production was almost the same quantity as the reaction that contained more enzymes. Accordingly, 6 U/g was considered as the optimum enzyme concentration considering the enzyme cost and reaction time.

3.3.3. Effects of pH on levan biosynthesis

Every kind of enzyme can only perform their maximum reaction rate in certain pH because enzyme configuration and activity are sensitive to pH values. The reaction rate will decline when pH values deviate out of their optimum conditions. Thus, the pH of reaction mixtures plays an important role in levan production. The effect of pH on levan production was described in Fig. 3c. The highest levan production was obtained with the pH value of 6.0. The levan production decreased with the pH lower or higher than 6.0. The optimum pH values of most of the levansucrase were in the range of 5.0–6.5 (Belghith, Dahech, & Belghith, 2012; Hernandez et al., 1995). However, Rairakhwada et al. (2010) reported that the optimum pH was 8.0 for levan formation of levansucrase from *B. amyloliquefaciens*.

3.3.4. Effects of metal ions on levan biosynthesis

The effects of metal ions on levan production were investigated. As shown in Fig. 3d, Ca^{2+}, Na^{+} and K^{+} had a negligible influence on levan production. Mg^{2+} had an obvious role in promoting the synthetic reaction and the levan production increased about 15% compared to the control. In others words, Mg^{2+} could activate the levansucrase activity. Similar results were reported by Belghith et al. (2012). However, Cu^{2+}, Fe^{2+}, and Zn^{2+} were shown to inhibit levan formation distinctly. Moreover, the effect of Mg^{2+} concentration on levan production was also studied (Fig. 3e). The highest levan production was obtained with 50 mmol/L Mg^{2+}. Therefore, 50 mmol/L Mg^{2+} was chosen as a metal ion promoter to the levan biosynthesis by levansucrase from *B. methylotrophicus* SK 21.002.

3.3.5. Effects of temperature on levan biosynthesis

The effect of temperature on enzyme reaction has the following aspects: (1) like general chemical reaction, reaction rate increases with temperature rising before reaching the optimum temperature. (2) As enzymes are protein, its denaturation rate also increase with temperature rising. So, enzymes have their optimum catalysis reaction temperature. As shown in Fig. 3f, the highest levan production exhibited at around 37 °C after 24 h incubation time. The yield of levan decreased significantly when the temperature was above 45 °C. The optimum temperature of levan formation for *B. methylotrophicus* SK 21.002 of 37 °C is similar to that of levansucrase from *M. laevaniformans* (Park et al., 2003). Moreover, higher optimum temperature for levan formation of 50 °C was reported for levansucrase from *Bacillus* sp. TH4-2 (Ammar et al., 2002).

4. Levan biosynthesis

At the end, the levan was biosynthesized under the optimum conditions for different time. The time curve of the reaction was shown in Fig. 4. The highest production reached to 100 g/L after 16 h, and the yield is about 33%, which was higher than 36 g/L (yield 24%) for *B. polymyxa* (NRRL B-18475), 50 g/L (yield 23%) for *Z. mobilis* and 15 g/L (yield 30%) for *Erwinia herbicola*, respectively (Han & Clarke, 1990; Keith et al., 1991; Shih et al., 2005). Furthermore, the levan production was decreased slightly with the prolonged incubation time. This might be due to the result of the hydrolysis activity of the levansucrase. Another noteworthy part of the result was that at the beginning of the experiment, since free fructose appeared much more rapidly than levan, the hydrolysis of sucrose was the main reaction. After 6 h, the rate of producing fructose decreased while levan appeared to be synthesized at a faster rate.

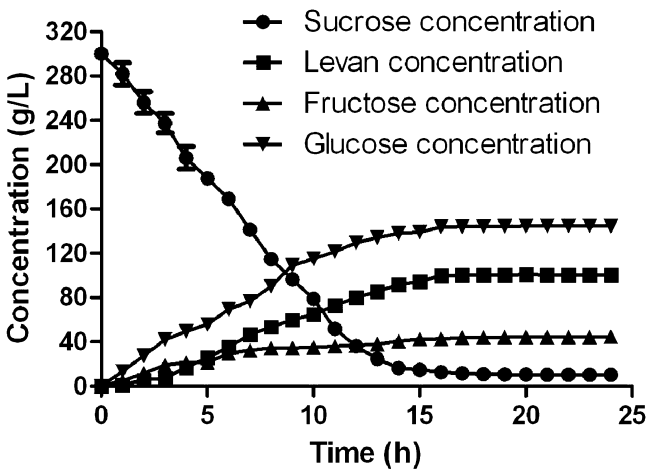

Fig. 4. The time course of product composition during levan biosynthesis with levansucrase under the optimal conditions (sucrose concentration 300 g/L, enzyme dosage 6 U/g sucrose, pH 6.0, 50 mM Mg^{2+}, and temperature 40 °C).

5. Conclusion

In this study, a novel strain, *B. methylotrophicus* SK 21.002, which could produce levansucrase, was isolated, characterized and identified. The ability to produce levan was confirmed by the levan purification and structure identification with sugar analysis, NMR and FT-IR. An effective biosynthesis method for producing levan was developed and the levan production reached about 100 g/L. Therefore, the levansucrase presented here could be considered as an outstanding potential candidate for future industrial applications. The investigation on the characteristics of levansucrase from *B. methylotrophicus* SK 21.002 is undertaking in our laboratory, which would be in favor of understanding the reaction mechanism and improving levan yield further.

Acknowledgments

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