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A recombinant levansucrase from *Bacillus licheniformis* 8-37-0-1 catalyzes versatile transfructosylation reactions

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ABSTRACT

This work disclosed the broad transglycosylation capability of the levansucrase from *Bacillus licheniformis* 8-37-0-1 for the first time. The levansucrase was firstly purified from the strain 8-37-0-1 and found to be a monomer of ~51 kDa with KETQDYKKSY as the *N*-terminus. Then, the gene encoding the enzyme was cloned and it contained an ORF of 1449 nucleotides, encoding a 482 amino-acid protein with a predicted 29 amino-acid signal peptide. The deduced mature protein without the signal showed the same *N*-terminus to the purified enzyme. The mature enzyme was subsequently expressed in *Escherichia coli*. The recombinant enzyme showed similar biochemical properties to the native one. It produced maximal yield of 7.1 mg/mL levan (M_r 9.6×10^6) from 0.8 M sucrose (pH 6.5) at 40 °C for 24 h *in vitro*. When using sucrose as the donor, the enzyme displayed a wide range of acceptor specificity and was able to transfer fructosyl to a series of sugar acceptors including hexose, pentose, β- or α-disaccharides, along with the difficult branched alcohols that have not been investigated before. Chemical structures of the resultant products were analyzed by MS and NMR spectra.

1. Introduction

Levansucrases (EC 2.4.1.10) catalyze fructosyl transfer following the mechanism of non-Leloir glycosyltransferases with sucrose as the main non-activated donor substrate. Such non-Leloir enzymes have great biotechnological potential, since they do not require expensively activated donors such as the nucleotide phosphosugars that are necessary for Leloir enzymes. The Gibbs binding energy released from hydrolysis of sucrose is available for saccharide formation [1].

Levansucrases are widely distributed in nature, especially abundant in gram-negative and gram-positive bacteria. They are of particular interest for the formation of levan, a β-(2→6)-linked fructose polymer with small amount of β-(2→1)-linked branch chains [2–4]. The degree of polymerization and type of branching are various depending on enzyme sources. These polymers possess wonderful physiological and biochemical characteristics and thus have broad applications. They can be widely used in food and non-food industries as viscosifier, stabilizer, emulsifier, gelling, or water-binding agent. Also, they have potential pharmaceutical applications owing to diverse bioactivities, such as antiviral, antitumor and immunostimulating activities [5–8].

In addition to levan, fructooligosaccharides (FOS) are also produced by some levansucrases *via* oligomerization from sucrose. Such kind of oligomers are described as healthful prebiotics, which exhibit low caloric values, decrease levels of lipids and cholesterol, help gut absorption of ions, and stimulate the *bifidobacteria* growth in the human colon [9]. Using sucrose as glycosyl donor, levansucrases can also catalyze fructosyl transfer to a wide range of acceptors including alcohols and mono- or oligosaccharides. The different sugar residues (*i.e.* galactose and xylose) that cap fructooligosaccharides may alter prebiotic and biochemical properties [10]. Some of the hetero-oligosaccharides have been found applications as sweeteners and as prebiotics [11]. Despite the promising prospect of developing fructosyl compounds with novel extended functions, the reported levansucrases are mostly focused on the formation of levan and FOS, while only a few of them have been fully characterized with respect to their acceptor specificity for transglycosylation [12–16].

In the previous work, a strain of *Bacillus licheniformis* 8-37-0-1 with high level of levan production (41.7 g/L) was isolated from the soil [17]. The levan product was subsequently purified from the fermentation broth and identified to be a novel polysaccharide, which contained a β-(2→6)-linked backbone with a single β-D-fructose at the C-1 position every seven residues, on average, along the main chain. Preliminary *in vitro* tests revealed it could significantly stimulate the proliferation of spleen lymphocyte [18].

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In this work, the levansucrase was purified from *B. licheniformis* 8-37-0-1, and the gene of the enzyme was cloned and over-expressed in *Escherichia coli*. The resulting recombinant enzyme was found to possess similar biochemical properties to the native one and thus was used in the synthesis of levan *in vitro*. Also, the enzyme was found to catalyze oligomerization reactions. It could efficiently transfer fructosyl to a lot of saccharides as well as novel branched alcohol acceptors. This is the first report of the powerful transglycosylation ability of the levansucrase from *B. licheniformis* 8-37-0-1. The excellent characteristics endowed the enzyme with a high capacity for obtaining novel fructosyl compounds and made it an alternative to the current synthetic enzyme sources.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

B. licheniformis 8-37-0-1 was cultured at 37 °C for 24 h in the medium (pH 7.0) containing 30 g/L sucrose, 1.0 g/L beef extract, 0.5 g/L $(NH_4)_2SO_4$, 2.5 g/L $K_2HPO_4{\cdot}3H_2O$, 2.5 g/L KH_2PO_4, 1.0 g/L NaCl, 0.2 g/L $MgSO_4{\cdot}7H_2O$, and 0.001 g/L $FeSO_4{\cdot}7H_2O$. *E. coli* strains DH5α and BL21 (DE3) were grown at 37 °C in LB medium. The pET-21b (+) plasmid (Novagen, US) was used for the construction of the expression vector with the His tag. The medium for the *E. coli* cells containing the pET-21b (+) plasmid was supplemented with ampicillin (100 μg/mL).

2.2. Enzyme and protein assays

The activity of levansucrase was measured by addition of enzyme solution (10 μL) to 40 μL of sucrose (finally at 0.1 M) in 50 mM phosphate buffer (pH 6.0). The reaction was performed at 37 °C for 60 min and then stopped by heating at 100 °C for 10 min. The resulting mixture was centrifuged and the glucose content in the mixture was analyzed using the glucose oxidase kit (Biosino Biotechnology and Science Inc., China). One unit of enzyme activity (U) is defined as the amount of enzyme that produces 1 μmol glucose per minute under the assay conditions. The concentrations of protein were measured according to Bradford method using BSA as the standard [19]. All enzyme and protein determinations were performed in triplicate.

2.3. Enzyme purification

All the procedures described below were performed at 4 °C. The culture of *B. licheniformis* 8-37-0-1 was centrifuged at 12,000 rpm for 5 min. The resultant supernatant was precipitated with ammonium sulfate at 80% saturation, followed by desalting. The sample was subsequently applied to a 1.1 × 20-cm DEAE Sepharose Fast Flow column (GE Healthcare, US) which had been pre-equilibrated with 50 mM phosphate buffer (pH 7.5). Then it was eluted by NaCl solution (pH 7.5) at concentration gradients from 0 to 0.4 M. The collected enzyme fragments were concentrated through centrifugal filters (Millipore, Germany) with a 30-kDa molecular mass cutoff.

2.4. Molecular mass determination

The molecular mass of levansucrase was determined by SDS polyacrylamide gel electrophoresis (SDS-PAGE) as well as gel filtration chromatography. Proteins in the polyacrylamide gel were visualized by Coomassie brilliant blue R-250 staining. Gel filtration chromatography was performed *via* a Superdex 200 (10 × 300 mm) column (GE Healthcare, US) pre-equilibrated with 150 mM NaCl in 50 mM phosphate buffer. Samples were eluted at a flow rate of 0.3 mL/min at 4 °C. The elution patterns were compared with those of the standard proteins, including ovalbumin (44 kDa), conalbumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa).

2.5. Gene cloning and heterogenous expression

Primers for the gene cloning were designed based on the sequence of the levansucrase from *B. licheniformis* DSM 13 (GenBank No. AE017333). The forward and reverse primers designed for the entire gene were 5′-CAGGTCGACATGAACACATCAAAAACATTGC-3′ and 5′-CGACTCGAGTTTGTTTACCGTTAGTTGTCC-3′ (*Sal* I and *Xho* I restriction sites are underlined), respectively. As for the gene without the signal-encoding sequence, the forward primer was 5′-CAGCATATGAAAGAAACGCAGGATTACAAG-3′ (*Nde* I site is underlined). The PCR reactions were performed in the presence of TaKaRa LA Taq polymerase, following the procedures including 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 2 min at 72 °C, and a final 5 min at 72 °C. PCR products were purified and sequenced.

For enzyme expression, the purified PCR products were subsequently digested by restriction enzymes, ligated onto the pET-21b (+) vector, and transformed into *E. coli* BL21 (DE3). The correct transformant was grown in LB medium at 37 °C, and the enzyme was induced by adding isopropyl-1-thio-β-D-galactoside (IPTG) when the cell density reached 0.6–1.0 at 600 nm. After continuous cultivation for three to 5 h, cells were harvested and disrupted by ultrasonic treatment. The lysate was centrifuged and the enzyme was purified from the suspension by Ni^{2+} chelation chromatography (Qiagen, Germany).

2.6. Characterization of native and recombinant levansucrase

Kinetic constants of native and recombinant enzymes were estimated by Lineweaver–Burk double reciprocal plots. Various concentrations of sucrose (0.1–1 M) were used to determine kinetic constants. Reactions were performed under assay conditions as described above. The effect of pH on the activity of the enzyme was determined by incubating the enzyme with 0.1 M sucrose in broad-range buffers containing 6.008 g/L citric acid, 3.893 g/L KH_2PO4, 1.769 g/L boric acid and 5.266 g/L barbitone and using NaOH to adjust the pH from 4.0 to 9.0. The effect of temperature was assayed at 20 °C to 65 °C. All assays were performed in triplicate.

2.7. Levan synthesis and analysis

Levan synthesis was carried out with the recombinant mature levansucrase in the presence of sucrose as substrate. The effects of sucrose concentrations were determined by incubating enzymes (50 U/mL) with sucrose solutions ranging from 0.1 M to 1 M in the pH 8.0 phosphate buffer at 37 °C for 24 h. The influence of the reaction time was assayed in 0.8 M sucrose at 37 °C. Aliquots were serially removed at 12 h intervals within 60 h period. The effects of temperature were assayed at 25, 30, 35, 40, 45, and 50 °C for 24 h. The impacts of pH on levan synthesis were performed in broad-range buffers with pH values ranging from 4.0 to 9.0 at 40 °C for 24 h. All the reactions were stopped by boiling at 100 °C for 10 min. Then the mixtures were centrifuged at 12,000 rpm for 5 min. Levan in the suspension was precipitated by 75% ethanol and quantified by the phenol-sulfuric acid method [17]. In the method, the polysaccharide was hydrolyzed by the concentrated sulfuric acid and released the monosaccharides that were quickly dehydrated, reacted with phenol and converted into colorimetric compounds. The absorbance of the compounds at certain wave length showed linear relationship with the sugar content in some range. A

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standard curve was firstly established between the concentration of fructose and the absorbance at OD490 after incubation with sulfuric acid and phenol. Then, the content of levan could be measured referring to the curve.

The homogeneity and molecular weight of the levan produced under the optimal condition were determined on a Waters High-Performance Size-Exclusion Chromatography (HPSEC) apparatus equipped with Two TSK columns, G3000 PWXL and G4000 PWXL in series, coupled to a refractive index detector (RID), and a Wyatt Technology Dawn-EOS Multi-Angle Laser Light Scattering detector (MALLS). The carrier solution was 0.2 M $NaNO_3$, and the samples were dissolved in 0.2 M $NaNO_3$ with stirring. The injection volume was 200 μL and the flow rate was 0.6 mL/min. The normalization of RID was conducted with bovine albumin monomer. The specific RI increment (dn/dc) at 658 nm and 25 °C was determined using an interferometric refractometer (Optilab/DSP, Wyatt Technology, USA). The dn/dc value was averaged to 0.135 mL/g and was assumed to be constant during the sample elution. ASTRA software (http://www.wyatt.com/) was utilized for data acquisition and analysis [18].

2.8. Oligosaccharide and fructoside synthesis

Using the sucrose as glycosyl donor, a series of mono-, di-, trisaccharides as well as sugar and aliphatic alcohols were tested as acceptors for transglycosylations. The reactions were performed at 40 °C for 24 h by incubation of the recombinant enzyme with 0.5 M sucrose and 0.5 M acceptors at pH 6.5, in the case of sugar or sugar alcohols as acceptors which included D-xylose, L-arabinose, L-rhamnose, D-mannose, D-galactose, melibiose, cellobiose, maltose, lactose, trehalose, sucralose, raffinose, dulcitol, sorbitol, mannitol and inositol. As for alkyl alcohols such as isopropanol, 1-pentanol, 1-hexanol, 1-octanol and 1-dodecanol, the reactions were operated under the same conditions except for the 10% (v/v) acceptor concentration. All the reactions were terminated by heating at 100 °C for 10 min. Sugar analysis was performed by TLC and HPLC as described below. The glycoside yields were calculated from the products using 50 U/mL enzymes based on the acceptor conversion.

2.9. Isolation of oligosaccharides and fructoside products

The oligosaccharide or glycoside products were concentrated by vacuum freeze dehydration. The resulting samples were loaded on preparative TLC 1-mm plates (Silica gel 60 F_{254}, Merck, Germany) using the developing solvent of *n*-butanol: ethanol: water (5:3:2, v/v/v) or subjected to a Bio-Gel P2 (Bio-Rad, US) column (1.5 × 100 cm) eluted by the deionized water for purification. The eluates were subjected to sugar determination by TLC, and the correct fractions were collected and lyophilized to dry powder.

2.10. TLC and HPLC analysis

TLC was performed with Silica gel 60 F_{254} plates (Merck, Germany). The developing solvent was a mixture of *n*-butanol: ethanol: water (5:3:2, v/v/v). Sugars on the TLC plate were detected by spraying with a solution of 0.5% (w/v) 3,5-dihydroxytoluene dissolved in 20% (v/v) sulphuric acid and subsequent heating at 120 °C for 5 min. HPLC was performed by Agilent 1200 series equipped with Agilent Zorbax carbohydrate analysis column (4.6 × 250 mm) and Agilent G1362A refractive index detector. Samples were eluted with acetonitrile: water (4:1, v/v) at a flow rate of 1.0 mL/min, with the column temperature maintained at 30 °C.

2.11. MS and NMR analysis

Mass spectra were recorded on a Shimadzu LCMS-IT-TOF instrument (Kyoto, Japan) equipped with an ESI source in positive/negative ion mode at a resolution of 10,000 full width at half-maximum. ^{1}H and ^{13}C NMR spectra were recorded at 26 °C with a Bruker DRX Advance-600 spectrometer (Bruker Biospin AG, Fallanden, Switzerland) at 600 MHz for ^{1}H and 150 MHz for ^{13}C. Chemical shifts were given in ppm downfield from internal TMS of D_2O. Chemical shifts and coupling constants were calculated from a first-order analysis of the spectra. Assignments were fully supported by homo- and hetero- nuclear correlated 2D techniques, including COSY (correlation spectroscopy), HSQC (hetero-nuclear single quantum coherence) and HMBC (hetero-nuclear multiple band correlation) experiments following standard Bruker pulse programs.

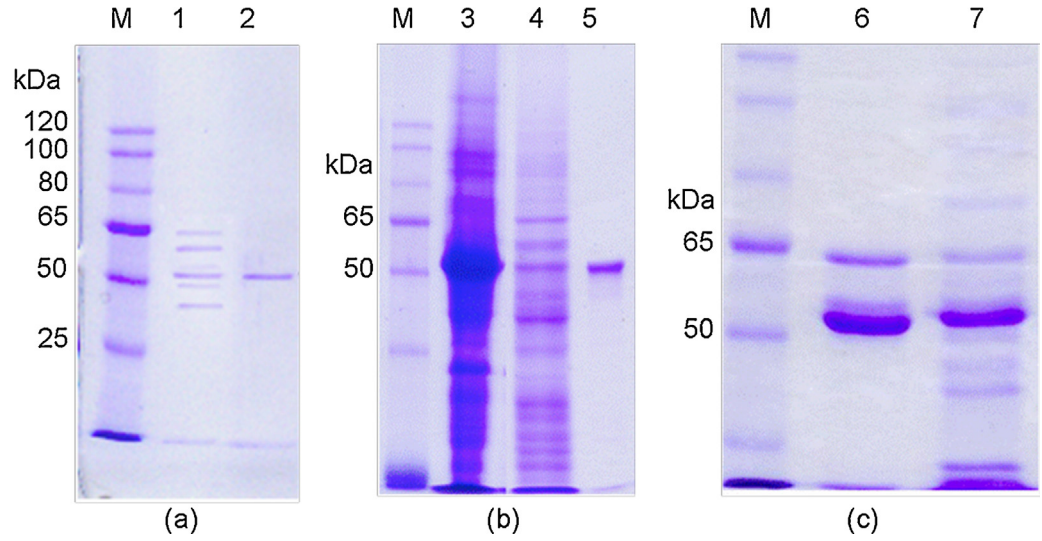

Fig. 1. SDS-PAGE analysis of native (a) and recombinant (b and c) levansucrase from *B. licheniformis* 8-37-0-1. M, marker proteins; lane 1, crude native enzyme from sulfate precipitation, lane 2, purified native enzyme; lane 3 and 4, crude enzyme from *sacB*-containing cells of *E. coli* with and without IPTG, respectively; lane 5, the purified recombinant SacB; lane 6, recombinant proteins from *sacB1*-containing cells of *E. coli*, which were purified through affinity and anion-exchange chromatography; lane 7, crude enzyme from *sacB1*-containing cells of *E. coli* in the presence of IPTG.

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2.12. Nucleotide sequence accession number

The GenBank accession number for the levansucrase gene of *B. licheniformis* 8-37-0-1 is KF647836.

3. Results and discussion

3.1. Purification, gene cloning and expression of levansucrase

The native levansucrase was purified from *B. licheniformis* 8-37-0-1 through ammonium sulfate precipitation, anion-exchange chromatography and ultrafiltration. The enzyme reached electrophoretic homogeneity with a molecular mass (M_r) of ~51 kDa as detected by SDS-PAGE (Fig. 1). The native form of the enzyme turned out to be a monomer since its natural molecular mass was less than 51 kDa as determined by gel filtration chromatography. It was in the range of the molecular masses (45–65 kDa) of mostly reported microbial levansucrases [15,20–26].

The purified enzyme in the SDS-polyacrylamide gel was subsequently electroblotted onto a polyvinylidene difluoride membrane. The protein band in it was cut out and sequenced by the method of Edman degradation. The *N*-terminal amino acid sequence was determined as KETQDYKKSY (Fig. S1).

Meanwhile, the gene of the levansucrase was obtained by PCR. It contained an open reading frame (ORF) of 1449 nucleotides, encoding a 482 amino-acid protein with a predicted M_r of ~53.7 kDa. Further analysis of the deduced protein by the software SignalP 4.0 revealed it had a putative signal peptide in the *N*-terminal region, with a predicted cleavage site between the 29th and 30th amino acids. The initial ten amino acids of the putatively mature protein which lacked the signal peptide were identical to those of the purified enzyme. Additionally, the theoretical M_r of the putatively mature protein (50.8 kDa) was consistent with the M_r of the purified enzyme (~51 kDa). These confirmed the actual existence of the signal peptide. The presence of the signal peptide seemed common in the extracellular levansucrase. The SacB enzymes from *B. subtilis* and *B. megaterium* were also found to bear a 29 amino-acid signal peptide in the reported literatures [21,27].

The nucleotide sequences of the entire and signal-deleted genes (designated as *sacB1* and *sacB*) of the levansucrase were ligated onto pET-21b (+) and transformed into *E. coli*, respectively. As shown in Fig. 1, either *sacB1* or *sacB* genes from *B. licheniformis* 8-37-0-1 was successfully expressed in *E. coli* cells. A protein band of ~52 kDa appeared in both kind of cells, while an additional protein band of ~57 kDa occurred only in the *sacB1*-containing cells and it could not be separated from the smaller one through affinity and anion-exchange chromatography. Thus, both of the protein bands were subjected to peptide mass fingerprinting. Two sequenced peptides (VMKPLIASNTVTDEIER and WYLFTDSR) were found to fully match the deduced levansucrase sequence from *B. licheniformis* 8-37-0-1 (Fig. S2). It proved the two proteins were actually the precursor and mature forms of a same enzyme. Similar phenomenon was previously observed when expressing *B. subtilis* levansucrase in *E. coli*, where appeared a mature enzyme of ~50 kDa along with its putative precursor of approximately 53 kDa [27]. As the *sacB*-containing cells only formed the mature protein that was comparable to the native purified protein, they were used for the production and purification of the recombinant SacB for the following analysis.

3.2. Characterization of the native and recombinant levansucrase

The Michaelis–Menten constants for native and recombinant SacB were consistent. The K_m values of them for sucrose were 140.81 and 152.79 mM, respectively, while the V_{max} values were equal to be about 0.03 mM/min. The K_m values of SacB were in the range of those of other microbial levansucrases (6.6–160.0 mM) [21,25,26,28–32].

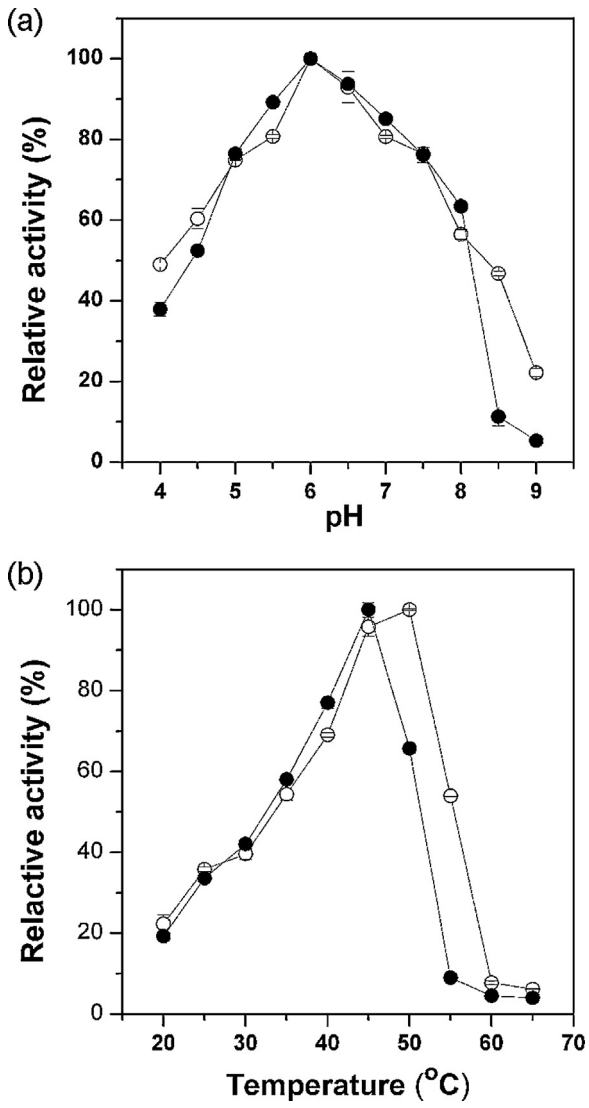

Fig. 2. The effect of pH (a) and temperature (b) on the activity of native (○) and recombinant (●) SacB.

The effects of pH on the activity of native and recombinant SacB were in similar patterns. Both enzymes were highly active at pH 6.0 and dropped by more than 50% activity at pH <4.0 or pH >8.5 (Fig. 2a). A lot of levansucrases were found to be of high activity at pH values near neutrality. For example, the enzyme from *B. megaterium* DSM319 presents an optimum range of pH 6.0–7.0. The hydrolysis activity significantly fell at pH values below 5.6 and above 7.6 [21]. Another example was that the highest enzyme activity from *L. mesenteroides* ATCC 8293 was found in the 6.5–7 pH range but with a rapid decrease in activity at pH >8 [33].

The optimal temperature for the activity of the recombinant SacB was 45 °C, slightly lower than that of the native enzyme (45–50 °C) (Fig. 2b). Both enzymes lost 80% activities at temperatures below 20 °C and were nearly inactive at above 60 °C. A number of reported levansucrases exhibited maximal activity at temperatures between 35 and 45 °C, including the enzymes from *L. mesenteroides* ATCC 8293 (35 °C) [32], *B. circulans* (45 °C) [11], *B. megaterium* DSM319 (45 °C) [21], and *Z. mobilis* UQM 2716 (45 °C) [29].

According to the above results of biochemical assays, only a few insignificant differences were found between the recombinant

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and native SacB from *B. licheniformis* 8-37-0-1. The recombinant enzyme had a negligible increase of K_m value as compared with the native one, which might be due to the introduced vector sequence that could slightly affect the affinity of the enzyme toward substrate. In addition, the native enzyme was a little more resistant for pH below 5.0 and beyond 8.0 as well as for temperatures above 45 °C. That might be related to the presence of native levan-enzyme complexes of which the substrate protection made the enzyme stronger [29]. Since the properties of the recombinant enzyme were generally in agreement with the native one, it was used as the enzyme source for the following transglycosylation reactions.

3.3. Synthesis of levan by the recombinant levansucrase

Levan synthesis was performed by incubation of the recombinant SacB with sucrose. Several efforts were directed toward evaluating the influence of various factors on the transglycosylation yield. These factors included substrate concentrations, reaction time, temperature and pH.

The effects of sucrose concentrations on levan synthesis were shown in Fig. 3a. The yield of levan was improved when the sucrose concentrations were increased from 0 to 0.8 M. The transferase activity of levansucrase was usually dependant on the sucrose concentration. An instance was that the enzyme from *B. amyloliquefaciens* showed enhanced transferase activity with the increased sucrose concentrations from 0.01 to 1.0 M [15]. Another instance was that the enzyme from *L. mesenteroides* ATCC 8293 displayed highly transferase activity at sucrose concentrations higher than 146 mM and retaining the activity up to a sucrose concentration as high as 875 mM [33].

Reaction time ranging from 0 to 60 h was tested (Fig. 3b). The levan yield was rapidly increased within the initial 24 h. When continuously extending the incubation time, the yields kept stable. Reaction time was seldom investigated as a factor involved in the levan formation. In this work, the initial incubation time of the enzyme with sucrose had a large influence on the amount of fructose transferred to levan. The levan yield at 12 h only achieved a half of the yield at 24 h.

The reaction temperature also exhibited significant impact on the levan production by the recombinant SacB (Fig. 3c). The yield of levan reached a maximum of 5.1 mg/mL at 40 °C and decreased at either lower or higher temperatures. It dropped to be around 2 mg/mL at 25 °C or 50 °C. The optimal temperature for levan synthesis (40 °C) was slightly lower than that for hydrolysis (45 °C). Similar phenomenon was observed in the enzyme from *B. circulans*, where the optimal temperature for transferase activity was 40 °C while for hydrolysis the highest initial rate extended to 45 °C [11]. As for the levansucrase from *Z. mobilis*, however, the situation was quite different. The sucrose hydrolysis was maximal at 45 °C whereas the levan-forming activity displayed the optimal activity at a low temperature of about 15 °C and it dropped to zero at 35 °C [29].

The pH affected the levan formation significantly (Fig. 3d). The yield reached the maximum of 7.1 mg/mL at pH 6.5, whereas it fell to around 4.0 mg/mL at pH 5.0 and 8.5. A number of levansucrases exhibited excellent activity for levan formation in the range of pH 5.5–8.0, such as the enzymes from *L. sanfranciscensis* TMW 1.392 (pH 5.5) [31], *L. mesenteroides* B-512 FMC (pH 6.2) [25], *P. syringae* NCPPB 1321 (pH 6.2) [28], *Geobacillus stearothermophilus* (pH 6.0–6.5) [16] and *B. amyloliquefaciens* (pH 8.0) [34].

Thus, the optimal conditions for the levan synthesis were an initial sucrose concentration of 0.8 M at pH 6.5 and 24 h incubation at 40 °C. The levan produced under the conditions was purified and its molecular mass was determined to be 9.6×10^6 by multi-angle laser light scatting, which was comparable to those formed by enzymes from *B. subtilis* (3×10^6) [21] and *L. sanfranciscensis* TMW 1.392 (5×10^6) [31] but significantly higher than that produced from native cells of *B. licheniformis* 8-37-0-1 (2.8×10^4) [18]. It was reported that the levans with high molecular masses were more effective for a direct effect on tumor cells, which is related to a modification in the cell membrane, including changes in cell permeability [35].

3.4. Oligosaccharide and fructoside synthesis by the recombinant levansucrase

In the presence of sucrose as glycosyl donor, the recombinant SacB from *B. licheniformis* 8-37-0-1 exhibited a wide range of acceptor specificity. It was able to catalyze fructosyl transfer to various sugars such as galactose, cellobiose, xylose, maltose, lactose, arabinose, and trehalose (Fig. S3), along with challengeable branched or long-chain alcohols like isopropanol and 1-pentanol. As a whole, the recombinant SacB exhibited better transglycosylation activity toward sugar acceptors than branched or long-chain alcohols from which only trace glycoside products were available (data not shown). As for sugar acceptors, the enzyme catalyzed the glycosyl transfer to maltose, arabinose, cellobiose, and xylose with high product yields of 37.5%, 36.8%, 34.7% and 31.5%. It showed moderate activity toward lactose with 25.8% glycoside yield and lower activity for galactose and trehalose with 10.1% and 8.5% glycoside yields.

The newly-produced glycosides were purified and subjected to MS analysis for the confirmation of fructosylations (Fig. S4). All of the derivatives from maltose, cellobiose, lactose and trehalose showed peaks of $[M+Na]^+$ ions in the MS spectra at m/z 527.15, consistent with fructosyl disaccharides (M_r 504). The galactose derivative displayed the peak of $[M+Na]^+$ ion at m/z 365.10, in agreement with fructosyl galactose (M_r 342). The signals of $[M+H_2O]^+$ ions at m/z 330.33 and 330.13 revealed the presence of fructosyl arabinose and xylose (M_r 312). The alkyl fructosides from isopropanol and 1-pentanol showed characteristic peaks of $[M+Na]^+$ ions 245.09 and 273.12, corresponding to isopropyl and pentyl fructosides (M_r 222 and 250), respectively.

Furthermore, transglycosylation products from maltose, cellobiose, lactose and xylose were analyzed by NMR for the structure information (Fig. S5). They were identified to be α-D-glucopyranosyl-(1 → 4)-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside, β-D-glucopyranosyl-(1 → 4)-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside, β-D-galactopyranosyl-(1 → 4)-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside, and α-D-xylopyranosyl-(1 → 2)-β-D-fructofuranoside. Their structures were shown in Fig. 4 and the relevant data were listed in Table 1. The fructose was added to all acceptors in β-(2 → 1) glycosidic bonds. The same phenomenon was also found in other levansucrase-catalyzed glycosylations toward sugar acceptors, such as the enzymes from *B. amyloliquefaciens* and *B. subtilis* NCIMB 11871 [12,15].

Levansucrases have already been known for their transferase activity when acceptors other than sucrose are added to the reaction medium. Nonetheless, the specificity of the enzymes toward acceptors differs considerably [11]. According to the product yields based on acceptor conversions, the enzyme from *B. amyloliquefaciens* showed higher activity toward disaccharides than monosaccharides [15]. In contrast, the enzyme from *B. subtilis* NCIMB 11871 fructosylated galactose and xylose more efficiently than disaccharides [12]. In this work, however, the acceptor specificity did not follow an obviously uniform principle. The common acceptors such as maltose, cellobiose, xylose, lactose and galactose were sequentially less efficient acceptors. Interestingly, the enzyme from *B. licheniformis* 8-37-0-1 showed high activity toward an unusual pentose, L-arabinose, which is a low-calorie

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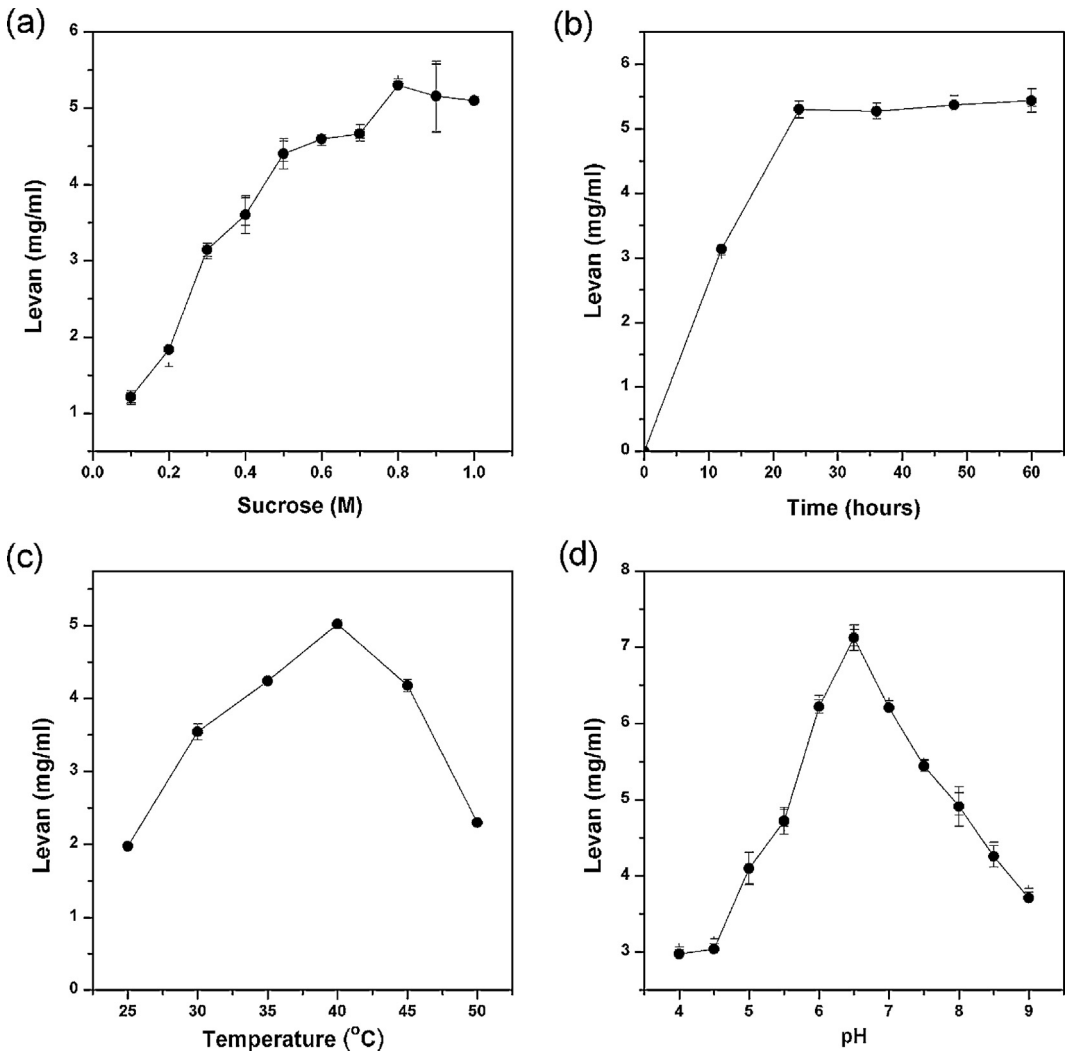

Fig. 3. The effect of substrate concentrations (a), reaction time (b), temperature (c) and pH (d) on the levan synthesis by the recombinant SacB.

Table 1
^{1}H and ^{13}C NMR data assignment for hetero-oligosaccharides produced by the recombinant SacB.

C-atom	Mal-Fru		Cel-Fru		Lac-Fru		Xyl-Fru	
	δC	δH	δC	δH	δC	δH	δC	δH
C-1	99.6	5.25	102.4	4.38	102.6	4.32	92.2	5.21
C-2	71.4	3.43	73	3.17	70.8	3.4	70.8	3.4
C-3	72.7	3.53	75.2	3.36	77.7	3.56	72.6	3.55
C-4	69.1	3.27	69.3	3.27	68.3	3.78	69	3.48
C-5	72.4	3.58	75.7	3.34	72.2	3.52	61.5	3.56
C-6	59.8	3.68	60.4	3.77–3.59	62.2	3.66	–	–
C-1′	91.8	5.24	91.7	5.25	91.6	5.25	60.5	3.52
C-2′	70.6	3.43	70.6	3.45	70.6	3.46	103.7	–
C-3′	72.8	3.87	70.9	3.72	71	3.74	75.8	4.09
C-4′	76.4	3.54	78.1	3.54	75.1	3.59	73.4	3.97
C-5′	70.7	3.81	70.9	3.82	70.9	3.83	81.2	3.75
C-6′	60.2	3.70–3.61	59.2	3.76–3.71	59.2	3.75–3.71	61.6	3.67–3.56
C-1″	61	3.51	61.1	3.52	61.1	3.52	–	–
C-2″	103.4	–	103.5	–	103.4	–	–	–
C-3″	76.2	4.05	76	4.06	76.1	4.06	–	–
C-4″	73.8	3.87	73.8	3.88	73.8	3.89	–	–
C-5″	81.1	3.73	81.2	3.74	81.2	3.74	–	–
C-6″	62.1	3.65	62.2	3.66	60.9	3.64–3.61	–	–

sweetener with promising application to reduce weight and control diabetes. The compound could selectively inhibit intestinal sucrase in an uncompetitive manner and suppress the increase of blood glucose [36]. Fructosylation of arabinose might expand its function to be as prebiotics besides as sweeter and anti-diabetic agent.

It was also interesting to note that the enzyme from *B. licheniformis* 8-37-0-1 was able to modify unusual alcohols such as

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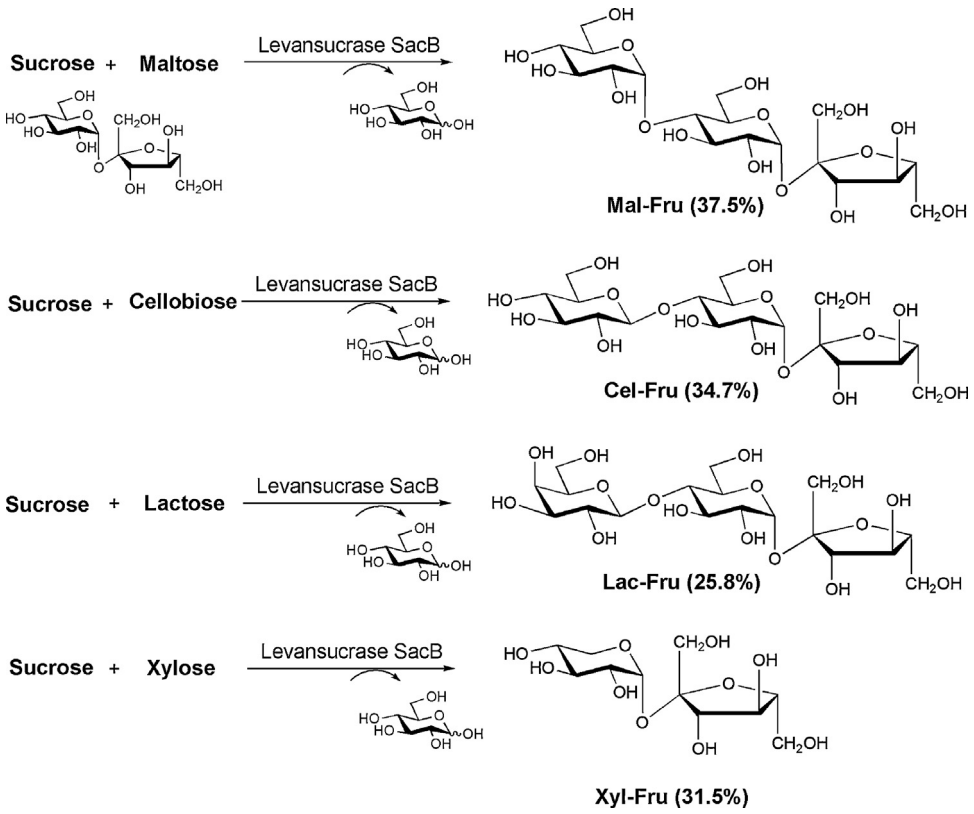

Fig. 4. Structures of hetero-oligosaccharides formed by the recombinant SacB. Mal-Fru, Cel-Fru, Lac-Fru and Xyl-Fru represent the products from maltose, cellobiose, lactose and xylose, respectively.

isopropanol and 1-pentanol, forming novel alkyl glycosides. Such kind of glycosides are widely used as biosurfactants in manufacturing of cosmetics and household chemicals as well as building blocks in pharmaceutical industry and molecular biology. In the case of levansucrase-mediated reactions, the known aliphatic-alcohol acceptors were limited to primary alcohols with less than four carbon atoms. The enzyme from *B. circulans* was reported to glycosylate methanol and butanol [11,37]. In this work, the recombinant SacB from *B. licheniformis* 8-37-0-1 exhibited transferase activity toward secondary alcohol like isopropanol, which was difficult for glycosylation due to steric hindrance even by chemical method. Additionally, it also glycosylated 1-pentanol with one carbon longer chain than the reported butanol. When continuously extending the chains to hexyl, octyl, and dodecyl alcohols, the enzyme showed no transferase activity. This might be related to the increasing hydrophobicity of those acceptors in one aspect, which made them unavailable to the hydrophilic enzyme.

In conclusion, the levansucrase derived from *B. licheniformis* 8-37-0-1 seemed to be a powerful catalyst for fructosylation and other synthetic applications. It showed excellent polymerization activity toward sucrose and displayed a wide range of acceptor specificity for oligomerization. The enzyme may be useful not only for the efficient synthesis of levan which shows significant biological, pharmaceutical, hygienical or industrial values, but also for the expansion of the repertoire of fructose-containing chemicals that could either possess inherent values by themselves or act as intermediates for further modification as valuable products.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2014.05.012.

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