Cloning and Expression of Levansucrase Gene of Bacillus velezensis BM-2 and Enzymatic Synthesis of Levan

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Abstract: Levan is a versatile and valuable fructose homopolymer, and a few bacterial strains have been found to produce levan. Although levan products have numerous specific functions, their application and promotion were limited by the production capacity and production cost. Bacillus velezensis BM-2 is a levan-synthesizing strain, but its levan production is too low to apply. In this study, the levansucrase gene of B. velezensis BM-2 was cloned to plasmid pET-32a-Acma-zz, and the recombinant plasmids were transferred to Escherichia coli BL21. A transformed clone was selected to express and secrete the fusion enzymes with an Acma-tag efficiently. The expressed products were further purified by a self-developed separating material called bacterial enhancer matrix (BEM) particles. The purification efficiency was 93.4%, with a specific activity of 16.589 U/mL protein. The enzymatic reaction results indicated that the optimal reaction temperature is 50 °C, the optimal pH of the acetate buffer is 5.6, and the buffer system greatly influenced the enzyme activity. The enzyme activity was enhanced to 130% in the presence of 5 mM Ca^{2+}, K^{+}, Zn^{2+}, and Mn^{2+}, whereas it was almost abolished in the case of Cu^{2+} and Fe^{3+}. The values of \( K_m \), \( k_{cat} \), and \( k_{cat}/K_m \) were 17.41 mM, 376.83 s^{-1}, and 21.64 mM^{-1}s^{-1}, respectively. The enzyme amount of 20 U/g sucrose was added to the system containing 400 g/L sucrose, and the levan products with a concentration of 120 g/L reached after an incubation of 18 h, which was 8 times that of the yield before optimization. The results of molecular docking analysis indicated that the Asp86 might act as a nucleophilic catalytic residue for sucrose, Arg246 and Asp247 act as transition state stabilizer of transfructosylation, and Glu340 and Arg306 were recognized as general acid donors. They formed the catalytic-groups triad. The unique properties and catalytic activity of the levansucrase suggest that it deserves further research and might have good industrial application prospects.

Keywords: clone; expression; B. velezensis; levansucrase; levan synthesis

1. Introduction

Levan, a common homopolysaccharide, is composed of fructose units predominantly by \( \beta-(2\rightarrow6) \) linkages, making the polysaccharide a unique carbohydrate polymer. Levan is produced by plants and microorganisms, including many algal cells, yeasts, fungi, and bacteria [1,2]. Previous studies have suggested that levan can be involved in biological stress resistance in bacteria and plants, such as modulating the osmotic pressure of cells and improving drought resistance, salinity resistance, and low-temperature protection [3–5]. Besides, levan has broad applications in many fields due to its functional and vital properties. Jakob et al. found that levan forms a hydrocolloid microgel, which can be used as an ingredient in wheat bread to extend the shelf life of products [6]. Tamer I. M. Ragab et al. suggested that levan could play a fundamental role in solving peptic ulcer problems [7]. Several other studies have confirmed that levan has prebiotic characteristics [7–9] and antioxidative [10], antiobesity [11], antifungal [12], antidiabetes [13], and antitumor [14] effects. Because of the excellent properties and application prospects of levan, it is urgent for researchers to improve its yield and quality.
Researchers have reported that levansucrase from microbial sources can use sucrose as a substrate to synthesize levan [15,16]. Levansucrase (EC 2.4.1.10), fructosyltransferase, belongs to the glycosyl hydrolase 68 (GH 68) family and “GH-J” clan and distributes in a wide range of microorganisms. Levansucrase catalyzes the hydrolysis of sucrose into glucose and fructose, the first fructose acceptor during levan biosynthesis is another sucrose molecule, thus creating the trisaccharide 6-kestose, the next fructosyl moiety is then transferred to the acceptor 6-kestose, and so on [17]. Levansucrases from Bacillus licheniformis [18], Bacillus subtilis [19], Brenneria goodwinii [16], Bacillus amyloliquefaciens [20], lactic acid bacteria [21], and Clostridium acetobutylicum [22] have been identified and can produce levan. Researchers have not reported on the levansucrase from B. velezensis in detail, including its gene information and enzymatic properties. Different microbial sources play distinct roles in the formation and activity of levansucrase, which could affect the size, branches, and biological activity of the polysaccharide [23–25]. To meet the needs of levansucrase research and its ultimate application, it is necessary to screen more microbial resources.

However, the wild-type strain produces only a small amount of levansucrase, which is difficult to isolate and purify, so the study of the enzyme is seriously hindered. The use of genetic engineering technology allows us to rapidly screen enzyme-producing strains from candidate bacteria and realizes the efficient expression of enzymes, which is a handy way to study the enzymatic properties and catalytic mechanism [16].

In this study, we aim to express heterologously levansucrase of B. velezensis BM-2 in E. coli and purify it by a unique method of our laboratory, bacterial enhancer matrix (BEM) particle one-step immobilization-purification, which is an environmentally friendly, simple, and low-cost technique for purifying enzymes. The study also sought to obtain its enzymatic property, kinetic properties, bioinformatics analysis, and optimization of levan production, and to explore the catalytic mechanism and its capacity to synthesize levan to evaluate the possible applications in industries.

2. Materials and Methods

2.1. Microorganisms and Culture Conditions

The levansucrase-producing bacterial strain used in this study was isolated from Chinese Dajiang and preserved at −80 °C in 30% (v/v) glycerol, at the Institute of Chemical Engineering, Tianjin University. Based on morphological characterization and 16S rDNA sequence, it was identified as B. velezensis and named BM-2. The strain was cultured in a medium containing 300 g/L sucrose, 5 g/L yeast extract, 10 g/L tryptone, 1 g/L K2HPO4, 10 g/L NaCl, and 1.47 g/L CaCl2. E. coli DH5α was used as an intermediate host for the construction of integrated plasmids. E. coli BL21 (DE3) was used as a recipient for expression. E. coli strains DH5α and BL21 (DE3) were grown at 37 °C in LB medium. The pET-32a-Acma-zz plasmid was used to construct an expression vector with the Acma-tag, which was designed and constructed in our laboratory [26,27]. The medium for the E. coli cells containing the pET-32a-Acma-zz plasmid was supplemented with ampicillin (100 µg/mL).

2.2. Plasmid Construction

BM-2 cells were cultivated and genomic DNA was extracted by a Genomic DNA Purification Kit (Tiangen Biotech). The BM-2 levansucrase gene was amplified by polymerase chain reaction (PCR). The coding region of the levansucrase gene (GenBank accession number: MN984684.1) was amplified using the genomic DNA of BM-2 as a template and the primers forward, 5′-CGCGAGCTCAAGAAAATACCCAAAAACC-3′ and reverse, 5′-CGCGAATTTCGTTGTAACCGTAAGCTGTC-3′ with Sacl and EcoRI restriction sites (underlined). The PCR products were digested with Sacl and EcoRI and inserted into pET-32a-Acma-zz that had been digested with the same enzymes to produce the pET-32a-levansucrase-Acma recombinant plasmid. The pET-32a-levansucrase-Acma plasmid was transformed into E. coli BL21 (DE3) for protein expression assays.
2.3. Expression of Levansucrase-Acma

The recombinant plasmid was transformed into *E. coli* BL21 (DE3) competent cells. For levansucrase expression, *E. coli* BL21 (DE3) harboring pET-32a-levansucrase-Acma was grown in LB broth containing 100 mg/L ampicillin at 37 °C. When the optical density value at 600 nm reached 0.6, the culture was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 16 °C for 18 h. The cells were lysed by ultrasonication, the cell debris were centrifuged at 10,000×g at 4 °C for 20 min [22,28], and the obtained supernatant contained the crude enzyme that was purified further.

2.4. Preparation of BEM Particles and Purification of Levansucrase-Acma by BEM Particles

*Bacillus* (*Bacillus subtilis*) was cultivated to a stationary phase (10–16 h). The bacterial cells were collected by centrifugation at 10,000×g at 4 °C for 15 min, resuspended in 0.1 M HCl, and boiled for 30 min to denature the DNA and proteins. The treated bacterial cells were cooled, recentrifuged to remove impurities (10,000×g, 4 °C, 30 min) twice in a washing buffer of 50 mM Tris-HCl (pH 7.2), and stored at 4 °C (10 mL of BEM particles prepared from 100 mL *Bacillus*). Equal parts of BEM particle sediment and crude enzyme (redissolved in 50 mM Tris-HCl at pH 7.2) were mixed at 25 °C for 30 min, and after recentrifuging (8000×g, 4 °C, 3 min), the precipitate was purified as levansucrase-Acma while the supernatant was labeled a control. The purification capacity and molecular weight were estimated by SDS–polyacrylamide gel electrophoresis.

2.5. Enzyme Assay

The levansucrase activity was measured by adding 40 µL purified levansucrase-Acma to 1 mL sodium acetate buffer (pH 5.6) with 100 g/L sucrose. The reaction was conducted at 35 °C for 30 min, and then the reaction was stopped, and reducing sugars were determined via the DNS method [29]. One unit of enzyme activity (U) was defined by calculating the amount of enzyme that released 1 µmol reducing sugars from sucrose per minute under the assay conditions. The protein concentration was measured according to the Bradford method using BSA as the standard.

2.6. Effects of Temperature, pH, and Metal Ions on Enzyme Activity

The effect of temperature on levansucrase-Acma enzyme activity was measured in a sodium acetate buffer (pH 5.6) at different temperatures (20–60 °C) for 30 min. A sodium acetate buffer (pH 3.8–5.8) and sodium phosphate buffer (pH 5.8–8.0) were used to study the effect of pH on levansucrase-Acma enzyme activity at 35 °C for 30 min. To determine the effects of various metal ions (Ca$^{2+}$, Cu$^{2+}$, Mg$^{2+}$, Fe$^{3+}$, Ba$^{2+}$, K$^+$, Zn$^+$, Ni$^{2+}$, and Mn$^{2+}$), these different metal ions (5 mM and 50 mM) were added to the reaction system (pH 5.6) and incubated at 35 °C for 30 min. The enzymatic activity in the absence of supplemental metal ions was regarded as 100%.

2.7. NaCl Tolerance of the Enzyme

A salinity test was conducted by incubating levansucrase-Acma with NaCl at different concentrations (0–4.0 M) for 30 min, and then the residual activity was calculated under the standard assay conditions. The reaction without NaCl was used as a blank control and defined as 100% enzyme activity.

2.8. Determination of Kinetic Parameters of Levansucrase-Acma

The kinetics of the levansucrase-Acma reaction were determined at different sucrose concentrations of 15.6, 31.3, 62.5, 125, 250, and 500 mM at 50 °C for 30 min. The enzymatic reactions were started by adding 40 µL purified levansucrase-Acma. The concentrations of released reducing sugar were determined via the DNS method. The $K_m$ (Michaelis constant) and $k_{cat}$ (turnover number) values were calculated using the standard Michaelis–Menten equation. All calculations were performed using Microsoft Office Excel.
2.9. Optimization of Levan Synthesis

2.9.1. Levan Synthesis and Detection

The phenol sulfuric acid method was used to determine polysaccharides [29,30]. Forty microlitres of purified levansucrase-Acma were incubated in 1 mL of 100 g/L sucrose in acetic acid and sodium acetate buffer (pH 5.6) at 50 °C for 2 h with shaking at 150 rpm. The reaction mixture was centrifuged (8000 × g, 4 °C, 3 min), then three volumes of precooled 95% ethanol were added to precipitate the levan in the resultant supernatant at 4 °C overnight; the interfering sugars were removed by ethanol [22]. The precipitate was collected by centrifugation (10,000 × g, 4 °C, 20 min) and redissolved in ultrapure water, and levan was determined using the phenol sulfuric acid method. Furthermore, the reaction with 40 µL BEM was used as a blank control. In this method, the polysaccharide was hydrolyzed by concentrated sulfuric acid and released monosaccharides that were quickly dehydrated, adsorbed by phenol, and converted into chromogenic compounds. The density of the compounds at certain wavelengths showed a linear relationship with the sugar content in some ranges. A calibration curve was established between the concentration of fructose at approximately 0.004–0.04 mg/mL and the optical density at 490 nm after incubation with sulfuric acid and phenol; then, the content of levan could be measured according to the curve [31].

2.9.2. Optimization of Enzyme Dose, Sucrose Concentration, and Reaction Time for Levan Synthesis

The optimal enzyme dose for levan synthesis was determined by incubating purified BM-2 levansucrase-Acma from 1 to 25 U/g sucrose in 1 mL sodium acetate buffer with 100 g/L sucrose (pH 5.6, 50 °C, 2 h).

To determine the optimum substrate concentration of levansucrase-Acma, the assay was carried out by using an enzyme dose of 20 U/g sucrose with different sucrose concentrations ranging from 100–600 g/L (pH 5.6, 50 °C, 2 h).

To determine the optimal reaction time for levan synthesis, the experiments were set up in a 1 mL sodium acetate buffer with 400 g/L sucrose from 0–24 h using an enzyme dose of 20 U/g sucrose (pH 5.6, 50 °C).

2.10. Statistical Analysis

All assays were performed in triplicate. Data management and analysis were performed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). All data are presented as the mean ± standard error of the mean.

2.11. 3D Prediction and Molecular Docking Investigations

Homologous modeling of levansucrase was performed with the SWISS-MODEL server (http://swissmodel.expasy.org (accessed on 28 December 2020)) and used the crystal structure of \(B.\) \(subtilis\) levansucrase (PDB ID: 3byl) as a template [32]. The Autodock 4.2.6 software package was introduced to remove water and hydrogenate proteins. Sucrose was utilized as the docking ligand, and structure energy minimization was implemented by Discovery Studio software (Accelrys, CA, USA) to eliminate unreasonable contacts. The structures were rendered with PyMOL Molecular Graphics System and Discovery Studio software (Accelrys, CA, USA) Visualizer.

3. Results and Discussion

3.1. Expression, Purification, and Sequence Analysis of Levansucrase-Acma

Although several levansucrases have been identified, detailed information about the gene sequence encoding \(B.\) \(velezensis\) levansucrase needed to be further investigated. In this work, the \(B.\) \(velezensis\) BM-2 levansucrase gene was expressed in \(E.\) \(coli\) using pET-32a-Acma as a vector. The BM-2 levansucrase gene was encoded by 1428 bp nucleotides, and the enzyme was a polypeptide chain formed by the dehydration of 444 amino acids. Then the amino acid sequence of the BM-2 levansucrase gene was compared that reported for
the enzyme from other microorganisms. The results showed that the BM-2 levansucrase shared a high identity with levansucrase from *B. atrophaeus* 1942 (87%), *B. licheniformis* (76%), and *B. megaterium* DSM 319 (82%) (Figure 1).

![Figure 1. Sequence alignment of levansucrases from *B. velezensis* BM-2 and other levansucrases. Sequences were compared with the following enzyme/source/accession numbers: levansucrase, *Bacillus atrophaeus* 1942 (GenBank: ADP33953.1); levansucrase, *Bacillus licheniformis* (GenBank: AC15886.1); levansucrase, *Bacillus megaterium* DSM 319 (GenBank: ADF38395.1); fructosyltransferase, *Lactobacillus* LTH5448 (GenBank: CAH25436.2). The alignment was conducted using ClustalX and ESPript software. The strictly conserved areas are shown with a red background, and the highly conserved residues are shown using red words surrounded by a blue box. However, a fairly low sequence identity (40%) was observed between *L. reuteri* LTH5448 fructosyltransferase and BM-2 levansucrase, it is well-known that the product synthesized by BM-2 levansucrase is a levan-type fructan [15], which might be attributed...
to the conserved amino acids in potential active sites to catalyze the transfer of fructose or bind the substrate. Moreover, multiple sequence alignment of levansucrase showed that most of the amino acids in the catalytic centers of levansucrases and transfructosylase are strictly conserved. Strong conservative amino acid residues play an important role in determining protein 3D structure and function among homologous proteins. Meanwhile, it could induce the loss and alteration of enzyme activity loss or alteration once codon mutations occur [33].

The gene fragment encoding the mature protein was expressed in *E. coli* BL21 (DE3) cells harboring the construct pET-32a-levansucrase-Acma plasmid, and 0.5 mM IPTG was used to produce high levels of the enzyme. Levansucrase-Acma was purified by modified BEM particle one-step immobilization-purification [26]. In previous work, the Gram-positive enhancer matrix (GEM) particles prepared by boiling the cells of *Lactococcus lactis* NZ9000 exerted an excellent purification ability [34]. In the present study, a more comfortable, more affordable, and more accessible method was performed by replacing *L. lactis* NZ9000 with common *Bacillus*. The resulting products were named BEM particles. In brief, BEM particles are nonliving particles obtained by boiling freshly grown *E. coli* in 0.1 M HCl for 30 min to remove the DNA and most proteins, followed by extensive washing with 50 mM Tris-HCl (pH 7.2). The BEM particles can be used to realize the one-step immobilization-purification of levansucrase-Acma because they consist of peptidoglycan substantially, linking a recombination protein with the Acma-tag in the C-terminus. Due to fusion with an Acma-tag, the recombinant levansucrase was conveniently purified by BEM particles one-step immobilization-purification. The purified enzyme showed a single protein band at approximately 53.5 kDa on SDS-PAGE (Figure 2), which was consistent with the predicted value, indicating that the target recombinant enzyme was successfully expressed in *E. coli*. Several protein bands were in the lane of the crude enzyme from recombinant *E. coli*, but there were no bands in the lane containing the BEM sample. The protein concentration of the purified levansucrase enzyme mixture was determined to be 0.1025 ± 0.0021 mg/mL, the final yield of pure protein obtained was approximately 10.3 mg from 100 mL *Bacillus subtilis*. The final purification efficiency of BM-2 levansucrase-Acma was 93.4%, with a specific activity of 16.589 U/mL protein, while the specific activity of the crude enzyme was 29.116 U/mL protein.

Protein purification is an essential step in biology. Among numerous purification methods, Ni-NTA chromatography is widely applied [16,22]. We have illustrated the steps of BEM purification (Figure 3), which can see the BEM approach is a time-saving
and simple strategy in protein purification [26], which can be equally verified by the data analysis in this study.

![Image of protein purification steps](image)

**Figure 3.** Illustration of bacterial enhancer matrix (BEM) particles one-step immobilization-purification.

3.2. Effects of Temperature, pH, Metal Ions and NaCl Concentration on Enzyme Activity

The effects of temperature, pH, different metal ions (5 mM, 50 mM) and NaCl on levansucrase-Acma activity were examined. Increasing temperature helps enhance substrate solubility and improve enzyme production efficiency in many reactions [35]. As shown in Figure 4a, levansucrase-Acma showed the highest activity at 50 °C, and the enzyme showed greater than 70% activity from 35 °C to 55 °C. These results indicate a slightly higher optimal temperature than those of *L. reuteri* LTH5448 levansucrase and *Bacillus methylotrophicus* SK 21.002 levansucrase [15,36], which may be attributed to the bacterial growth environment in which much heat was produced during the bean paste fermentative process. However, levansucrase-Acma activity decreased sharply at 60 °C; perhaps the high temperature resulted in enzyme inactivation and ceased all productive activity.

![Image of enzyme activity at different temperatures](image)

**Figure 4a.** Enzyme activity at different temperatures.

The effect of pH on levansucrase activity was measured at 35 °C. Figure 4b indicates the relationship between the release of levansucrase-Acma reducing sugars at different pH values; over 80% activity was displayed from pH 5.2 to 5.8, and acetate buffer at pH 5.6 was optimal for levansucrase-Acma. Remarkably, the buffer system greatly influenced the enzyme activity. The activity was 37-fold higher in acetate buffer at pH 5.8 than that in sodium phosphate buffer. In addition, BM-2 levansucrase-Acma activity exhibited a rise–fall trend with increasing pH in the sodium phosphate buffer, while the optimum pH for levansucrase was found to be 5.2–6.2 in the other levansucrases reported, despite their enzyme activity range being pH 5.8–8.0 [16,31,37]. It can be seen that pH has a significant effect on levansucrase activity from different sources.

![Image of enzyme activity at different pH values](image)

**Figure 4b.** Enzyme activity at different pH values.

To determine the effect of metal ions on enzyme activity, 5 and 50 mM of metal ions were added to levansucrase-Acma reaction mixtures with sucrose as the substrate. The reaction without any metal ions was used as a blank control. As shown in Figure 4c, at the low concentration (5 mM), Ca^{2+}, K^+, Zn^{2+}, and Mn^{2+} can increase the catalytic activity above 130% compared with the initial relative activity, which is different from a previous report of levansucrase that only Ca^{2+} contributed to improving the enzyme activity [15]. No studies have reported that levansucrase requires a metal cofactor, although a low-affinity Ca^{2+} binding site has been mapped to Thr265 in Bs-levansucrase [38]. The enzyme activity was almost unaffected by Mg^{2+}, Ba^{2+}, and Ni^{2+}. Compared with the low concentration, the high concentration (50 mM) of almost all the metal ions could significantly decrease the enzyme activity. These results indicated that the advancement of metal ion concentration may restrain levansucrase-Acma activity. Notably, 5 and 50 mM Cu^{2+} and Fe^{3+} both had an evident inhibitory influence on enzyme activity, so Cu^{2+} and Fe^{3+} should be avoided in actual production. Thus, these results suggested that the enzymatic reaction...
of levansucrase-Acma could introduce suitable cationic cofactors that promote the release of glucose.

Figure 4. Enzymatic properties of the recombinant BM-2 levansucrase-Acma. (a) The effect of temperature. (b) The effect of pH on the catalytic activity. Two different buffer systems were used in the analysis: sodium acetate buffer (pH 3.8–5.8) and sodium phosphate buffer (pH 5.8–8.0). (c) The effect of various metal ions (5 mM, 50 mM). (d) The effect of NaCl concentration.

The reaction without NaCl was used as a blank control and defined as 100%, and enzymatic reactions were carried out under different NaCl concentrations (Figure 4d). With increasing NaCl concentrations in the range of 0–3.6 M, greater than 80% catalytic activity of levansucrase-Acma was still observed. This result suggested that levansucrase-Acma possesses certain NaCl tolerance. However, there was an obvious decreasing trend at 4.0 M NaCl, even causing the enzyme activity to decrease to 0, which might be attributed to the high concentration of NaCl, which altered the enzyme structure and inactivated levansucrase-Acma. Li et al. discovered that the NaCl concentration could affect protein folding; the protein had a fully folded structure at high concentrations and was restricted to combining with the substrate, and a higher salt concentration might increase the solvent density and viscosity by suppressing diffusion of the protein [39]. Recently, researchers attempted to evaluate the impact of NaCl concentration on levansucrase. Faten A. [17] found that A. awamori levansucrase was halophilic at 3.0 M NaCl and that the spores isolated from the osmophilic medium of honey can produce an enzyme with halophilic properties. Onur K. [28] reported that the levansucrase (HsLsc) from Halomonas smyrnensis AAD6 T showed maximum activity in the presence of 3.5 M NaCl. These results pointed to a firm relationship between the isolates’ bio-characteristics and their origin [17,28].

3.3. Kinetic Parameters

The kinetic parameters of BM-2 levansucrase-Acma were estimated using the double reciprocal plot method (Lineweaver-Burk plots) at the optimal temperature (50 °C) for the highest activity. The resulting kinetic parameters are illustrated in Table 1. The values of $k_{\text{m}}$, $k_{\text{cat}}$ (turnover number), and $k_{\text{cat}}/K_{\text{m}}$ (catalytic efficiency) were determined to be 17.41 mM, 376.83 s$^{-1}$ and 21.64 mM$^{-1}$s$^{-1}$, respectively. The affinity of levansucrase for
sucrose varies among microorganisms. The $K_m$ of this enzyme was lower than that of lsc-3 from *Pseudomonas syringae* (18.5 mM) [40], levansucrase from *Erwinia tasmaniensis* (50.5 mM) [41], and *B. licheniformis* 8-37-0-1 WT (33.3 mM) [31]. Low $K_m$ values indicated a high affinity of the enzyme-substrate complex [42]. Levansucrase from *B. licheniformis* RN-01 [18] and *B. licheniformis* 8-37-0-1 WT [43] showed a low turnover number ($k_{cat}$) for both reactions, resulting in low catalytic efficiency ($k_{cat}/K_m$). Although the levansucrase of *E. tasmaniensis* had a higher $k_{cat}$ (497.9 s$^{-1}$), it had only a low $k_{cat}/K_m$ (9.86). These results suggested that BM-2 levansucrase may be used to generate levan efficiently with sucrose.

Table 1. Kinetic parameters of levansucrase in comparison with other organisms.

|                          | *B. velezensis* Levansucrase-Acma | *Erwinia Tasmaniensis* Levansucrase | *B. Licheniformis* RN-01 LsRN | *Pseudomonas Syringae* lsc-3 | *Bacillus Licheniformis* 8-37-0-1WT Levansucrase |
|--------------------------|-----------------------------------|-------------------------------------|-------------------------------|--------------------------------|-----------------------------------|
| $K_m$ (mM)               | 17.41                             | 50.5                                | 7.14                          | 18.5                           | 33.3                              |
| $k_{cat}$ (s$^{-1}$)     | 376.83                            | 497.9                               | 64.0                          | 504.4                          | 159.9                             |
| $k_{cat}/K_m$ (mM$^{-1}$s$^{-1}$) | 21.64                            | 9.86                                | 8.96                          | 27.3                           | 4.8                               |

3.4. Optimization of Enzyme Dose, Sucrose Concentration, and Reaction Time on Levan Synthesis

To obtain a high yield of levan for the BM-2 levansucrase-Acma, some important process parameters, such as enzyme dose, sucrose concentration, and reaction time, were optimized.

Figure 5a shows that at a certain temperature and pH, the yield of levan was improved when the enzyme amount was increased from 1 to 13 U/g sucrose. Subsequently, levan synthesis had a slowly increasing trend and reached a maximum yield of 19 g/L when using 20 U/g sucrose levansucrase. Then, a fluctuant reduction in levan yield was observed, followed by an increase in the enzyme amount. This result demonstrated that the amount of levansucrase was saturated. The excess enzymes might intend to compete with the water and sucrose in the reaction system, resulting in a partial stop of the transfructosylation.

To make full use of the substrate and improve production efficiency, the effect of sucrose concentration on levan synthesis is exhibited in Figure 5b. The yield of levan was significantly improved when the sucrose concentration increased from 100 to 400 g/L under optimized conditions. The optimum sucrose concentration for levan synthesis (37 g/L) of BM-2 levansucrase-Acma was found to be 400 g/L, and a further increase in the sucrose concentration of the reaction system decreased levan synthesis. Sucrose concentration generally plays a substantial role in the production of levan. Our results were similar to those of previous studies [15,16,31], and all these results indicated that an appropriately high concentration favors the production of levan. Nonetheless, a high sucrose content may lead to a high viscosity of the reaction system, which is detrimental to molecular diffusion, making the enzyme unable to exchange and catalyze the reaction more efficiently.

Levan was produced using the optimized reaction conditions (50°C, pH 5.6, 20 U/g sucrose, 400 g/L sucrose). As shown in Figure 5c, the course may be divided into three stages: quick rise stage, slow increment stage, and plateau stage. The yield of levan increased rapidly during the initial 6 h, and after an 18 h reaction, the highest levan synthesis was obtained at 120 g/L, which was approximately 8 times the yield before optimization. As the reaction time increased, the production of levan decreased slightly; a possible explanation for this is the partial hydrolysis of levan [16,37]. The levan yield varies greatly among levansucrases from different types of microorganisms. *Halomonas* levansucrase could produce 18 g/L levan after optimization [44], levansucrase from recombinant *Saccharomyces* could produce 76 g/L levan [45], *B. licheniformis* 8-37-0-1 levansucrase produced a maximal yield of 7.1 mg/mL levan [32], and *Brenneria goodwinii* levansucrase reached a yield of 185 g/L levan [16]. In this investigation, the levan yield of BM-2 levansucrases
was higher than those reported in most of the studies; accordingly, the enzyme might be more suitable for industrial production.

![Graph](image-url)

**Figure 5.** Optimization of enzyme dose, sucrose concentration, and reaction time on levan synthesis. (a) The effect of enzyme dose on levan synthesis. (b) The effect of sucrose concentration on levan synthesis. (c) The effect of reaction time on levan synthesis.

### 3.5. Molecular Docking Investigations

Previous reports indicated that molecular modeling was an appropriate way to propose the exact binding sites [46]. To computationally determine the binding affinity and the preferred orientation of sucrose when it was bound to *B. velezensis* BM-2 levansucrase, molecular docking was carried out. The BM-2 levansucrase model was built by SWISS-MODEL using the crystal structure of *B. subtilis* levansucrase (PDB ID: 3byl) as a template, which had a high sequence identity of 89.64% with BM-2 levansucrase. The score of the GMQE item is 0.94, the GMQE score is expressed as a number between 0 and 1, and higher numbers indicate higher reliability. As shown in Figure 6a, five-antiparallel \( \beta \)-sheets coupled with several loops formed a \( \beta \)-propeller, which is the main part of the overall structure of the *B. subtilis* levansucrase model, and a central negatively charged...
cavity formed a funnel located at the bottom of the β-propeller. The structure is typical of GH68 [22]. The funnel may host the active site and sucrose recognition residues. Most likely, the funnel bottom could provide a channel for sucrose access to the active pocket, and the levan chain extends from the funnel neck when holding the growing chain near the entrance of the active site, which contributes to growing chain elongation, therefore, increasing the chance of sucrose re-entering the enzymatic pocket.

The sucrose docked well into the putative active pocket (Figure 6b), catalytic triplex groups composed of the amino acid residues Asp86, Arg246 and Asp247, and Glu340 and Arg360. We hypothesize that these residues are analogous to the catalytic triad of the “ping-pong replacement mechanism”, including the transition state stabilizing residue, the acid/base residue, and the nucleophile residue, respectively. Arg246 and Asp247 act as transition state stabilizer of transfructosylation and have favorable interactions with glucose and fructose, implying their potential function in stabilizing the reaction. Glu340 and Arg306 interact with fructose and are recognized as a general acid donor. Asp86 is speculated to act as a nucleophilic catalytic residue for sucrose, which forms hydrogen bonds with lengths of 2.4 Å with the polar hydroxyl hydrogen of sucrose. This unique combination contributes to the formation of a stable levansucrase-sucrose compound. In addition, the levansucrases from other bacteria exhibited a catalytic triad, such as Asp135, Asp 309, and Glu401 of the levansucrase from G. diazotrophicus [47]. Researchers evaluated the impact of some important residues at binding sites by site-directed mutation. When Asp68, Asp225, and Glu309 in Brs-LS were replaced by alanine, extreme activity loss was observed [48]. A possible explanation for this might be that these three amino acids may not be the only catalytic amino acids involved in the active pocket. We speculate that the catalytic-group is a combination of regional amino acids rather than individual amino acids, which could be explored for further research in the future.

Figure 6. Active pocket for sucrose docking to levansucrase. (a) Structural alignment of the predicted 3D structures and molecular docking of BM-2 levansucrase using the crystal structure of B. subtilis levansucrase (PDB ID: 3byl) (green) as a template. Cartoon representation of BM-2 levansucrase structure with a view into the funnel, sucrose as the ligand molecule shown as a stick model with the carbon skeleton marked with pink and the hydroxyl group marked with red. (b) Magnified view of the molecular docking of the catalytic active cavity of the black box in Figure 6A. Details of the BM-2 levansucrase contacts in the substrate bond complex, coupling with the nucleophilic, transition stabilizer, and general acid residues. The selected residues Asp86 (green), Asp247 (green), Arg246 (green), and Glu340 (green) and Arg360 (green) were predicted to localize to the surface of the active cavity. The dashed green line is the hydrogen bond with the value of the bond length.
4. Conclusions

Levan is widely used in chemistry, cosmetics, medicine, and the food industry due to its unique properties. Nevertheless, the production capacity and production cost limit the application and promotion of levan. In this study, we reported the expression and characterization of recombinant BM-2 levansucrase compared with other reported levansucrases, and the optimal conditions for levan biosynthesis were identified. This study not only offered enzymatic characteristics but also provided insight into the molecular structure of BM-2 levansucrase. Based on multiple sequence alignment, some strictly conserved sequence motifs were found that may determine enzyme 3D structure and function. Molecular docking demonstrated the dominant combination of enzyme and ligand, as well as speculated that Asp86, Arg246 and Asp247, and Glu340 and Arg360 formed a catalytic-groups triad, further enhancing our understanding of the levansucrase and sucrose interaction. Overall, the BM-2 levansucrase could be a promising candidate for the industrial production of levan, and this study contributes to better use of levansucrase. Further work would enable us to determine the chemical modification, site-directed mutation, biological cogeneration, or other innovative technologies to investigate further scaling up of levan synthesis.

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References

1. Cerning, J. Exocellular polysaccharides produced by lactic acid bacteria. *FEMS Microbiol. Rev.* 1990, 7, 113–130. [CrossRef][PubMed]
2. Öner, E.T. Microbial Production of Extracellular Polysaccharides from Biomass. In *Pretreatment Techniques for Biofuels and Biorefineries*; Fang, Z., Ed.; Springer: Berlin/Heidelberg, Germany, 2013; pp. 35–56. [CrossRef]
3. Dong, C.X.; Zhang, L.J.; Xu, R.; Zhang, G.; Zhou, Y.B.; Han, X.Q.; Zhang, Y.; Sun, Y.X. Structural characterization and immunostimulating activity of a levan-type fructan from *Curcuma kwangsiensis*. *Int. J. Biol. Macromol.* 2015, 77, 99–104. [CrossRef][PubMed]
4. Versluys, M.; Kirtel, O.; Öner, E.T.; Van den Ende, W. The fructan syndrome: Evolutionary aspects and common themes among plants and microbes. *Plant. Cell Environ.* 2018, 41, 16–38. [CrossRef]
5. Matsuhira, H.; Tamura, K.-i.; Tamašiake, H.; Sato, Y.; Anzai, H.; Yoshida, M. High production of plant type levan in sugar beet transformed with timothy (Phleum pratense) 6-SFT genes. *J. Biotechnol.* 2014, 192, 215–222. [CrossRef][PubMed]
6. Jakob, F.; Pfaff, A.; Novoa-Carballoa, R.; Rubsam, H.; Becker, T.; Vogel, R.F. Structural analysis of fructans produced by acetic acid bacteria reveals a relation to hydrocolloid function. *Carbohydr. Polym.* 2013, 92, 1234–1242. [CrossRef]
7. Ragab, T.I.M.; Shalaby, A.S.G.; Awdan, S.A.E.; El-Bassyouni, G.T.; Salama, B.M.; Helmy, W.A.; Esawy, M.A. Role of levan extracted from bacterial honey isolates in curing peptic ulcer: In vivo. *Int. J. Biol. Macromol.* 2020, 142, 564–573. [CrossRef]
8. Porras-Dominguez, J.R.; Avala-Fernández, A.; Rodríguez-Alegria, M.E.; Miranda-Molina, A.; Escalante, A.; González-Cervantes, R.; Olivera, C.; López Munguía, A. Levan-type FOS production using a *Bacillus licheniformis* endolevanase. *Process. Biochem.* 2014, 49, 783–790. [CrossRef]
9. Adamberg, K.; Adamberg, S.; Ernts, K.; Larionova, A.; Voor, T.; Jaagura, M.; Visnapuu, T.; Alamoe, T. Composition and metabolism of fecal microbiota from normal and overweight children are differentially affected by melibiose, raffinose and raffinose-derived fructans. *Anaerobe* 2018, 52, 100–110. [CrossRef]
10. Dahech, I.; Harrabi, B.; Hamden, K.; Feki, A.; Mejdoub, H.; Belghith, H.; Belghith, K.S. Antioxidant effect of nondigestible levan and its impact on cardiovascular disease and atherosclerosis. *Int. J. Biol. Macromol.* 2013, 58, 281–286. [CrossRef]
11. Oh, J.; Lee, S.R.; Hwang, K.T.; Ji, G.E. The anti-obesity effects of the dietary combination of fermented red ginseng with levan in high fat diet mouse model. *Phytother. Res.* **2014**, *28*, 617–622. [CrossRef] [PubMed]

12. Song, B.; Zhu, W.; Song, R.; Yan, F.; Wang, Y. Exopolysaccharide from *Bacillus vallismortis* WF4 as an emulsifier for antifungal and antiarthritic peppermint oil emulsion. *Int. J. Biol. Macromol.* **2019**, *125*, 436–444. [CrossRef]

13. Dahech, I.; Belgithi, K.S.; Hamden, K.; Feki, A.; Belgithi, H.; Mejdoub, H. Antidiabetic activity of levan polysaccharide in alloxan-induced diabetic rats. *Int. J. Biol. Macromol.* **2011**, *49*, 742–746. [CrossRef]

14. Yoo, S.H.; Yoon, E.J.; Cha, J.H.; Lee, H.G. Antitumor activity of levan polysaccharides from selected microorganisms. *Int. J. Biol. Macromol.* **2004**, *34*, 37–41. [CrossRef] [PubMed]

15. Ni, D.; Xu, W.; Bai, Y.; Zhang, W.; Zhang, T.; Mu, W. Biosynthesis of levan from sucrose using a thermostable levanosucrase from *Lactobacillus reuteri* LTH5448. *Int. J. Biol. Macromol.* **2018**, *113*, 29–37. [CrossRef] [PubMed]

16. Liu, Q.; Yu, S.; Zhang, T.; Jiang, M.; Mu, W. Efficient biosynthesis of levan from sucrose by a novel levanosucrase from *Brenneria goodwinii*. *Carbohydr. Polym.* **2017**, *157*, 1732–1740. [CrossRef]

17. Mostafa, F.A.; Abdel Wahab, W.A.; Salah, H.A.; Nawwarr, G.A.M.; Esawy, M.A. Kinetic and thermodynamic characteristic of *Aspergillus awamori* EM66 levanosucrase. *Int. J. Biol. Macromol.* **2018**, *119*, 232–239. [CrossRef] [PubMed]

18. Nakapong, S.; Pichyangkura, R.; Ito, K.; Iizuka, M.; Pongsawasdi, P. High expression level of levanosucrase from *Bacillus licheniformis* RN-01 and synthesis of levan nanoparticles. *Int. J. Biol. Macromol.* **2013**, *54*, 30–36. [CrossRef] [PubMed]

19. Wang, Y.; Chen, S.; Zhao, X.; Zhang, Y.; Wang, X.; Nie, Y.; Xu, Y. Enhancement of the production of *Bacillus naganoeensis* pullulanase in recombinant *Bacillus subtilis* by integrative expression. *Protein Expr. Purif.* **2019**, *159*, 42–48. [CrossRef]

20. Han, Y.; Li, E.; Liu, L.; Zhang, B.; Wang, Y.; Gui, M.; Wu, R.; Li, P. Rheological, emulsifying and thermostability properties of two exopolysaccharides produced by *Bacillus amyloquefaciens* LPL061. *Carbohydr. Polym.* **2015**, *115*, 230–237. [CrossRef]

21. Shi, Q.; Hou, Y.; Xu, Y.; Morkeberg Krogh, K.B.R.; Tenkanen, M. Enzymatic analysis of levan produced by lactic acid bacteria in fermented doughs. *Carbohydr. Polym.* **2019**, *208*, 285–293. [CrossRef]

22. Gao, S.; Qi, X.; Hart, D.J.; Gao, H.; An, Y. Expression and Characterization of Levanosucrase from *Clostridium acetobutylicum*. *J. Agric. Food Chem.* **2017**, *65*, 867–871. [CrossRef]

23. Ortiz-Soto, M.E.; Porras-Dominguez, J.R.; Seibel, J.; Lopez-Munguia, A. A close look at the structural features and reaction conditions that modulate the synthesis of low and high molecular weight fructans by levanosucrases. *Carbohydr. Polym.* **2019**, *219*, 130–142. [CrossRef] [PubMed]

24. Hill, A.; Chen, L.; Mariage, A.; Petit, J.-L.; de Berardinis, V.; Karbourne, S. Discovery of new levanosucrase enzymes with interesting properties and improved catalytic activity to produce levan and fructooligosaccharides. *Catal. Sci. Technol.* **2019**, *9*, 2931–2944. [CrossRef]

25. Kanjanathanin, P.; Pichyangkura, R.; Sithiyotha, T.; Charoenwongpaiboon, T.; Wangpaiboon, K.; Chunsririrost, S. Computational design of *Bacillus licheniformis* RN-01 levanosucrase for control of the chain length of levan-type fructooligosaccharides. *Int. J. Biol. Macromol.* **2019**, *140*, 1239–1248. [CrossRef]

26. Zhao, F.; Song, Q.; Wang, B.; Han, Y.; Zhou, Z. Purification and immobilization of the soluble and insoluble portions of recombinant lipase by gram-positive enhancer matrix (GEM) particles. *Int. J. Biol. Macromol.* **2019**. [CrossRef]

27. Xu, W.; Ni, D.; Yu, S.; Zhang, T.; Mu, W. Insights into hydrolysis versus transfructosylation: Mutagenesis studies of a novel levanosucrase from *Brenneria sp*. *EnBiD312*. *Int. J. Biol. Macromol.* **2018**, *116*, 335–345. [CrossRef]

28. Kirtel, O.; Menendez, C.; Versluys, M.; Van den Ende, W.; Hernandez, L.; Toksoy Oner, E. Levanosucrase from *Halomonas snymersis* AAD6(T): First halophilic GH-J clan enzyme recombinantly expressed, purified, and characterized. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 9207–9220. [CrossRef] [PubMed]

29. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356. [CrossRef]

30. Juan, L.U.; Min, X.; Lili, L.U. Optimization of Fermentation Conditions for Production of Levan by *Bacillus licheniformis* 8-37-0-1. *Food Sci.* **2011**, *32*, 183–187.

31. Lu, L.; Fu, F.; Zhao, R.; Jin, L.; He, C.; Xu, L.; Xiao, M. A recombinant levanosucrase from *Bacillus licheniformis* 8-37-0-1 catalyzes versatile transfructosylation reactions. *Process Biochem.* **2014**, *49*, 1503–1510. [CrossRef]

32. Meng, G.Y.; Futterer, K. Structural framework of fructosyl transfer in *Bacillus subtilis* levanosucrase. *Nat. Struct. Biol.* **2003**, *10*, 935–941. [CrossRef]

33. Xu, W.; Peng, J.; Zhang, W.; Zhang, T.; Guang, C.; Mu, W. Enhancement of the *Brenneria* sp. levanosucrase thermostability by site-directed mutagenesis at Glu(404) located at the “TEAP” residue motif. *J. Biotechnol.* **2019**, *290*, 1–9. [CrossRef] [PubMed]

34. Zhao, F.; Song, Q.; Wang, B.; Du, R.; Han, Y.; Zhou, Z. Secretion of the recombination alpha-amylase in *Escherichia coli* and purification by the gram-positive enhancer matrix (GEM) particles. *Int. J. Biol. Macromol.* **2019**, *123*, 91–96. [CrossRef]

35. Choi, H.J.; Kim, C.S.; Kim, P.; Jung, H.C.; Oh, D.K. Lactosucrose bioconversion from lactose and sucrose by whole cells of *Lactobacillus reuteri* LTH5448. *Int. J. Biol. Macromol.* **2019**, *125*, 436–444. [CrossRef]

36. Li, R.; Zhang, T.; Jiang, B.; Mu, W.; Miao, M. Purification and characterization of a novel intracellular levanosucrase derived from *Bacillus methylotrophicus* SK 21.002. *J. Biotechnol.* **2014**, *20*, 1876–1879. [CrossRef] [PubMed]

37. Peng, J.; Xu, W.; Ni, D.; Zhang, W.; Zhang, T.; Guang, C.; Mu, W. Preparation of a novel water-soluble gel from *Erwinia amylovora* levan. *Int. J. Biol. Macromol.* **2019**, *122*, 469–478. [CrossRef]
38. Petit-Glatron, M.F.; Grajcar, L.; Munz, A.; Chambert, R. The contribution of the cell wall to a transmembrane calcium gradient could play a key role in *Bacillus subtilis* protein secretion. *Mol. Microbiol.* 1993, 9, 1097–1106. [CrossRef]

39. Li, J.; Xie, Y.; Wang, R.; Fang, Z.; Fang, W.; Zhang, X.; Xiao, Y. Mechanism of salt-induced activity enhancement of a marine-derived laccase, Lac15. *Eur. Biophys. J.* 2018, 47, 225–236. [CrossRef]

40. Visnapuu, T.; Mardo, K.; Alamaee, T. Levansucrases of a *Pseudomonas syringae* pathovar as catalysts for the synthesis of potentially prebiotic oligo- and polysaccharides. *New Biotechnol.* 2015, 32, 597–605. [CrossRef]

41. Polsinelli, I.; Caliandro, R.; Salomone-Stagni, M.; Demitri, N.; Rejzek, M.; Field, R.A.; Benini, S. Comparison of the Levansucrase from the epiphyte *Erwinia tasmaniensis* vs its homologue from the phytopathogen *Erwinia amylovora*. *Int. J. Biol. Macromol.* 2019, 127, 496–501. [CrossRef] [PubMed]

42. Hamilton, L.M.; Kelly, C.T.; Fogarty, W.M. Purification and properties of the raw starch-degrading alpha-amylase of *Bacillus sp.* IMD 434. *Biotechnol. Lett.* 1999, 21, 111–115. [CrossRef]

43. He, C.; Yang, Y.; Zhao, R.; Qu, J.; Jin, L.; Lu, L.; Xu, L.; Xiao, M. Rational designed mutagenesis of levansucrase from *Bacilluslicheniformis* 8-37-0-1 for product specificity study. *Appl. Microbiol. Biotechnol.* 2018, 102, 3217–3228. [CrossRef]

44. Erkorkmaz, B.A.; Kirtel, O.; Duru, O.A.; Oner, E.T. Development of a cost-effective production process for Halomonas levan. *Bioprocess. Biosyst. Eng.* 2018, 41, 1247–1259. [CrossRef] [PubMed]

45. Ko, H.; Bae, J.H.; Sung, B.H.; Kim, M.J.; Kim, C.H.; Oh, B.R.; Sohn, J.H. Efficient production of levan using a recombinant yeast *Saccharomyces cerevisiae* hypersecreting a bacterial levansucrase. *J. Ind. Microbiol. Biotechnol.* 2019, 46, 1611–1620. [CrossRef] [PubMed]

46. Jafari, A.; Shareghi, B.; Hosseini-Koupaei, M.; Farhadian, S. Characterization of osmolyte-enzyme interactions using different spectroscopy and molecular dynamic techniques: Binding of sucrose to proteinase K. *Int. J. Biol. Macromol.* 2019. [CrossRef]

47. Martinez-Fleites, C.; Ortiz-Lombardia, M.; Pons, T.; Tarbouriech, N.; Taylor, E.J.; Arrieta, J.G.; Hernandez, L.; Davies, G.J. Crystal structure of levansucrase from the Gram-negative bacterium Gluconacetohacter diazotrophicus. *Biochem. J.* 2005, 390, 19–27. [CrossRef] [PubMed]

48. Xu, W.; Liu, Q.; Bai, Y.; Yu, S.; Zhang, T.; Jiang, B.; Mu, W. Physicochemical properties of a high molecular weight levan from *Brenneria sp.* EniD312. *Int. J. Biol. Macromol.* 2018, 109, 810–818. [CrossRef] [PubMed]