Heliyon 8 (2022) e12137

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Characterization of levansucrase produced by novel *Bacillus siamensis* and optimization of culture condition for levan biosynthesis

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ARTICLE INFO

Keywords:
Bacillus siamensis
Levan
Levansucrase
Prebiotics
Anion-exchange chromatography

ABSTRACT

Levan has attracted interest due to the potential health benefits associated with its prebiotic, biological, and functional properties. However, the production of levan is expensive due to its high resource requirements. With the growing demand for levan, it is vital to determine suitable cultivation condition for its production and reduce costs accordingly. The present study characterized the enzyme levansucrase produced by a novel strain of *Bacillus siamensis* and optimized the conditions for the biosynthesis of levansucrase and levan. The crude levansucrase enzyme production by *B. siamensis* was induced at a specific temperature in a medium containing different concentrations of sucrose, fructose, and glucose to evaluate transfructosylation and hydrolysis activities. Crude levansucrase significantly increased transfructosylation relative to hydrolysis activity at 37 °C in a medium containing 20% (w/v) sucrose. Both transfructosylation and hydrolysis activities were inhibited in glucose and fructose containing medium. Purification and characterization of the levansucrase were performed by precipitating the enzyme with ammonium sulfate solution, purified anion-exchange chromatography, and analyzed by Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The results showed the molecular weight of the enzyme to be approximately 30 kDa with specific activity at 15.95 U/mg, corresponding to a protein purification efficiency of 11.47 and a yield of 78.75%. The optimal culture condition for the purified-levansucrase activity for levan biosynthesis was obtained at 37 °C after 48 h, at pH 6.0 in 50 mM phosphate buffer and 20% (w/v) sucrose. The study demonstrated the optimized condition for levan biosynthesis utilizing the *B. siamensis* that can serve as a model for various commercial and industrial applications for efficient levan production.

1. Introduction

Levan-type fructans have been highly promising as fructan substitutes in the food industry due to their biological and functional properties. Levan possesses prebiotic properties that promote the growth of gut microbiota of the human gastrointestinal tract [1, 2, 3]. It is widely recognized that levan exhibits numerous properties important in various industries as viscosifier [4], stabilizer [5], emulsifier [6], gelling agent [7], and water-binding agent [8]. The broad range of pharmaceutical and medical applications of levan made it be a promising candidate as nanoparticle that enhances peptide delivery [9, 10], antitumor [11], anti-irritant [12], antioxidant, and anti-inflammatory agent [13].

The levan structure is composed of D-fructofuranosyl residues linked together by β-(2–6) linkages (the main chain) and some β-(2–1)-linked branched chains [14]. It is synthesized by the levansucrase enzyme (EC 2.4.1.10), a fructosyltransferase belonging to the glycoside hydrolase family 68 (GH68). This GH68 is found to be produced by several bacterial strains [15], including the species *Brenneria goodwini* [16], *Bacillus subtilis* [17], *Gluconobacter japonicus* [18], *Halomonas species* [19], *Klebsiella strain* [20], *Leuconostoc citreum* [21], and *Zymomonas mobilis* [22]. Levansucrase functions by catalyzing the conversion of sucrose to levan via the fructosyltransferase reaction. The active sites of enzymes determine their affinity for specific compounds and the ability for a reaction to occur. In the case of the levansucrase, its active site is composed of two subsites; one with a high affinity for fructose (−1 subside) and the other

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https://doi.org/10.1016/j.heliyon.2022.e12137
Received 26 February 2022; Received in revised form 26 May 2022; Accepted 28 November 2022

for available glucose and fructose residues (+1 subside). When sucrose binds to the levansucrase's active sites, it forms a covalent fructosyl-enzyme intermediate at −1 subside that is further hydrolyzed to release glucose. Another fructose residue on sucrose attracts the fructosyl-enzyme at the +1 subside, resulting in the synthesis of β-(2, 6)-linked levan [23].

Michaelis–Menten kinetics revealed that levansucrase catalyzes sucrose hydrolysis and transfructosylation [24]. The activity ratio of hydrolysis to transfructosylation is highly dependent on the initial reaction conditions used. Numerous reports indicate that a high sucrose concentration enhances transfructosylation activity at lower temperatures (between 4 °C and 40 °C) [14, 25]. While enhanced hydrolysis activity occurs optimally at temperatures above 50 °C [26]. With regards to pH, reports found that levansucrase is most effective at pH 5.0 and 7.0 but less so at very low and high pH [27, 28]. Numerous studies reported various factors such as enzyme concentration, temperature, ionic strength, and organic solvents influence the polymerization and molecular structures [29]. In the case of *Bacillus subtilis*, levansucrase favored the synthesis of high-molecular-weight levan (approximately 2000 kDa) at low enzyme concentrations (0.1 U/mL) [30]. Thermophilic *Bacillus* species produced high-molecular-weight levansucrase at elevated temperatures [31]. *Gluconobacter albidus*, however, when supplemented with sodium gluconate medium with levansucrase yielded a high molecular weight of approximately 10^6 kDa [32]. It is evident that various reaction parameters, such as pH, temperature, and sucrose concentrations, influence levansucrase activity, hence affecting levan biosynthesis, and these factors may vary between bacteria species.

Our previous study established that the isolated novel strain of *Bacillus siamensis* from fermented soybeans possesses biocatalytic properties and found to produce levansucrase. The *B. siamensis* strain produced a novel extracellular levansucrase for levan biosynthesis induced by using sucrose as the substrate [33]. With the aim to reduce production costs and to efficiently produce levan, the current study characterized the levansucrase enzyme produced by the novel strain of *B. siamensis* and optimized culture conditions for levansucrase production and levan biosynthesis.

2. Materials and methods

2.1. Bacterial strain

B. siamensis was previously isolated in our laboratory from fermented soybeans and was found to produce levan at high sucrose concentrations [33]. *B. siamensis* is a gram-positive rod bacterium that belongs to the *Bacillus* genus, which has members found throughout nature. *B. siamensis* has been gaining attention for its probiotic properties [34].

2.2. Culture medium

Pre-growth medium containing 50 g/L sucrose, 5 g/L yeast extract, 10 g/L tryptone, and 5 g/L NaCl was used for *B. siamensis* inoculation. Culture medium composed of 200 and 300 g/L sucrose, 3.5 g/L Na_2HPO_4, 0.8 g/L NaH_2PO_4, 0.2 g/L $MgSO_4$, 3.5 g/L $NaNO_3$, and 5 g/L yeast extract was used for levan biosynthesis according to our previous study [33].

2.3. Culture medium optimization for B. siamensis levansucrase production

The cultivation of *B. siamensis* was carried out in 250 mL sterilized Erlenmeyer flasks, and the subculture (10% inoculum) was added to the culture medium (200 mL final volume) containing 20% and 30% (w/v) sucrose, respectively. The pH was maintained at 6.0 using 50 mM phosphate buffer and was incubated for 24 h at 37 °C with shaking (200 rpm) in a shaker incubator. At 0, 6, 12, and 24 h, 3 mL of sample was collected and centrifuged at 9100 × g for 60 min. The pellet was washed twice with normal saline, and the optical density at OD600 was used to determine the cell concentration. The supernatant was analyzed using high-performance liquid chromatography (HPLC), and an enzyme assay was performed to determine the residual sugar concentration. The optimal temperature for cultivation was determined by culturing *B. siamensis* at various temperatures (25 °C, 30 °C, 37 °C, 45 °C, and 50 °C) with the optimal sucrose concentration at pH 6.0. Glucose and fructose monosaccharides were added at concentrations of 2%, 4%, 6%, 8%, and 10% (w/v), with the optimal sucrose concentration, temperature, and a pH of 6.0 (Table 1).

2.4. Purification and characterization of levansucrase

B. siamensis was cultured in a 2 L Erlenmeyer flask containing a 1 L culture medium at 37 °C, 200 rpm for 48 h. The supernatant was collected for enzyme purification using a DEAE Toyopearl 650M column. The column was pre-equilibrated with 50 mM phosphate buffer (pH 6.0). The supernatant was loaded onto the column, and protein was eluted at a flow rate of 1 mL/min using 500 mL of NaCl concentration (0–1.0 M NaCl) in 50 mM phosphate buffer (pH 6.0). The eluted fractions were harvested at a concentration of 5 mL per fraction. The protein and enzyme activities of each fraction were determined using the OD280 and levansucrase assays. The SDS-PAGE was performed to identify the protein molecular mass of the active fraction [35].

2.5. Culture condition optimization for levansucrase activity

The effect of varied concentrations of sucrose for levan production was determined by adding different sucrose concentrations, 5%, 10%, and 20% (w/v), by adding 3 mL of 0.6 U/mL levansucrase to 3 mL of each

Table 1. The cultivating condition of *B. siamensis* in the culture medium under agitation at 200 rpm and pH 6.0.

Experiment	Sugar contained in the medium (% (w/v))			Temperature (°C)	Time (h)
	Sucrose	Glucose	Fructose		
Effect of sucrose concentration	20, 30	-	-	37	24
Effect of temperature	20	-	-	25, 30, 37, 45 50	12
Effect of glucose concentration	20	2, 4, 6, 8, 10	-	37	12
Effect of fructose concentration	20	-	2, 4, 6, 8, 10	37	12

Table 2. The optimal conditions of levansucrase activity for levan production.

No.	Sucrose concentration (%)	Temperature (°C)	pH	Time (h)	Agitation (rpm)
Effect of sucrose concentration	5, 10, 20	37	6	24	200
Effect of pH (with acetate buffer)	20	37	4, 5, 6	3	200
Effect of pH (with phosphate buffer)	20	37	6, 7, 8	3	200
Effect of temperature	20	25, 37, 50	6	6	200

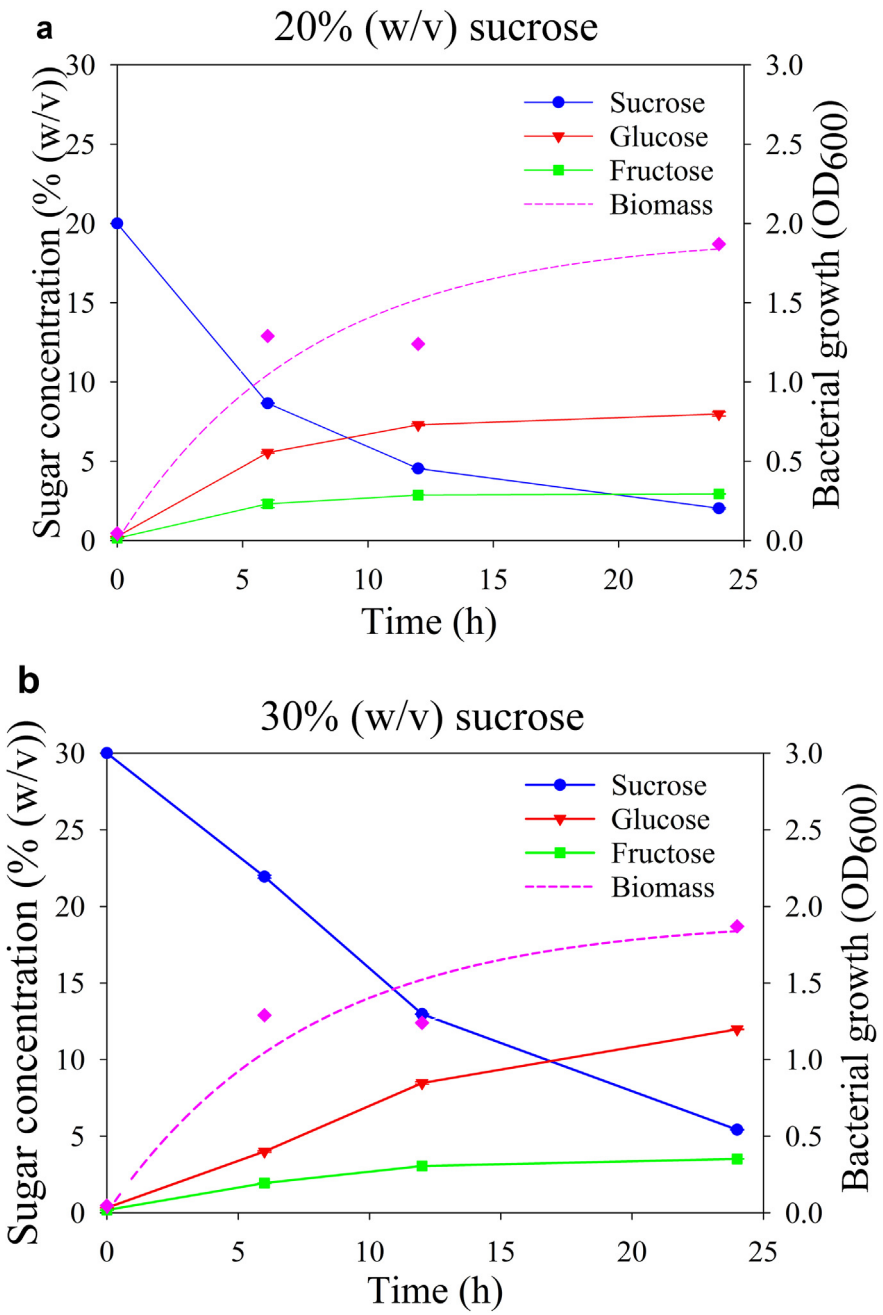


Figure 1. Bacterial growth kinetics of *B. siamensis* and sugar product profiles were obtained in 20% (w/v) sucrose (a) and 30% (w/v) sucrose (b) broth media with a pH 6.0 at 37 °C.

sucrose concentration in 50 mM phosphate buffer. The initial pH, temperature, and agitation speed were set to 6.0, 37 °C, and 200 rpm, respectively, and incubated for 48 h (Table 2).

HPLC was performed to determine the enzyme activities and levan concentration. The sucrose concentration that produced the highest activity was selected to evaluate the effect of pH on enzyme activity. Three milliliters of sucrose were mixed with 50 mM acetate buffer (to obtain pH of 4.0, 5.0, and 6.0) and 50 mM phosphate buffer (to obtain pH of 6.0, 7.0, and 8.0). The mixture was incubated at 37 °C and 200 rpm for 1, 3, and 6 h to determine the enzyme activity and levan production. While the sucrose concentration and pH resulting in the highest activity were fixed in the previous experiment, the effect of temperature was investigated by adding 3 mL of 0.6 U/mL levansucrase to the 3 mL sucrose with buffer. For 3 h, the mixtures were incubated at 25 °C, 37 °C, and 50 °C, at 200 rpm. HPLC was used to determine the enzyme activity and levan production. All samples were taken at appropriate time intervals and the sugar content was determined using HPLC [36, 37].

2.6. Analytical methods

2.6.1. Enzyme assay and protein determination

The enzyme activities were determined by adding 0.5 mL of the crude supernatant to 0.5 mL of 20% (w/v) sucrose in 50 mM phosphate buffer, pH 6.0. A shaker incubator was used to incubate the mixture for 2 h at 37 °C, at 200 rpm. The reaction was terminated by boiling at 100 °C for 10 min, and the residual sugar was determined using HPLC. One unit of crude enzyme activity is defined as the amount of levansucrase that produces 1 μmol of glucose per minute per mL of the enzyme [38]. The hydrolysis activity was estimated as the amount of free fructose (μmol) released per min per mL of the enzyme. The transfructosylation activity

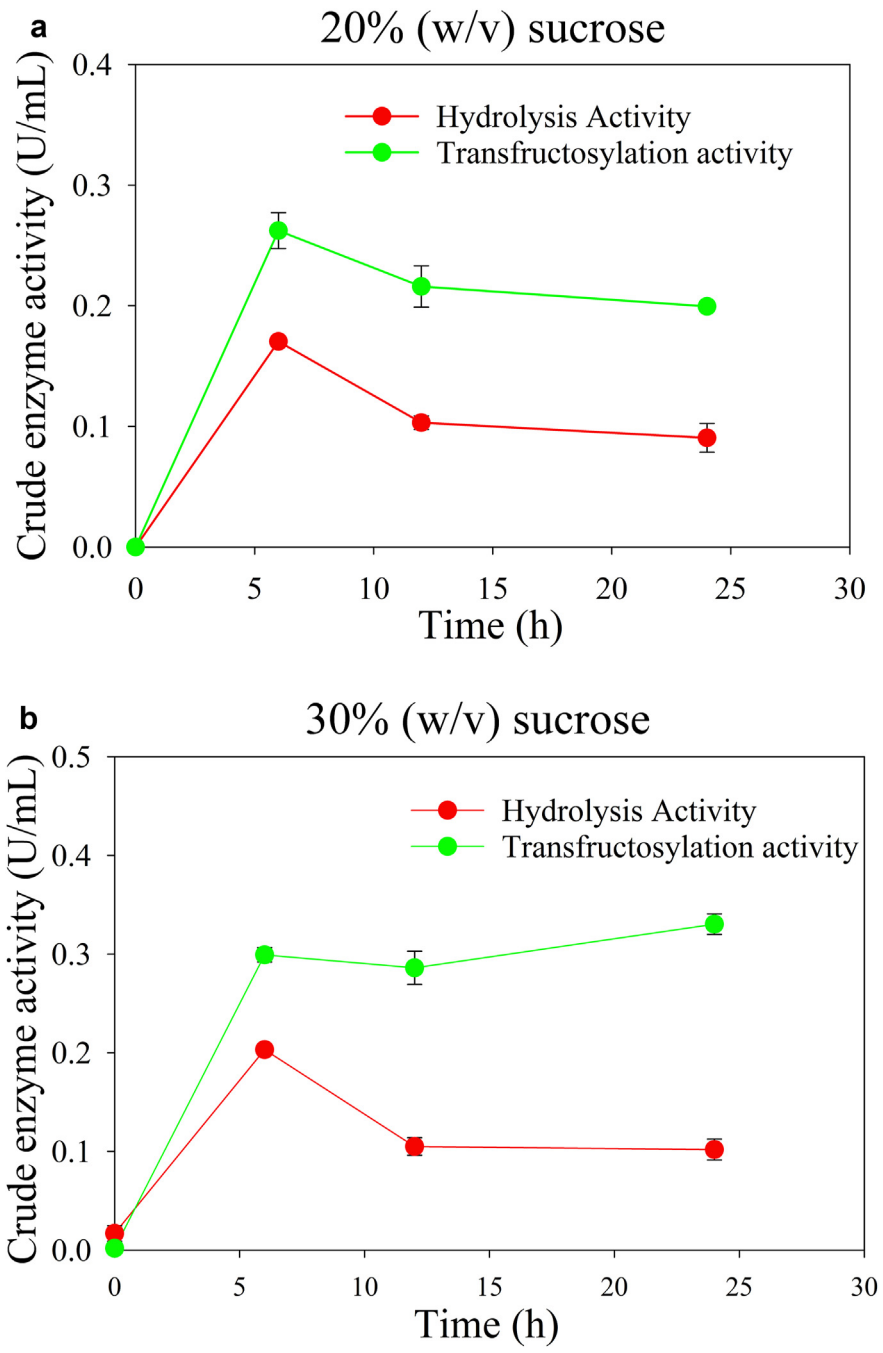


Figure 2. Effects of sucrose concentration, including 20% (w/v) sucrose (a) and 30% (w/v) sucrose (b), on the crude enzyme activity of *B. siamensis* with a pH of 6.0 at 37 °C.

was quantified as the μmol of fructose transferred to the enzyme acceptor molecule per min per mL of the enzyme [39]. The protein concentration was determined using the Bradford assay, as described elsewhere [40].

2.6.2. High-performance liquid chromatography (HPLC) analysis

The resulting sugar was analyzed using an NH_2 column and a refractive index (RI) detector, with acetonitrile/water mixtures serving as the eluent [36]. The sugar residual after incubation was determined using an NH_2 column (Luna®, 5 μm NH_2 100 Å, 250 × 4.6 mm) coupled to an HPLC instrument (Shimazu LC-20A series, Shimazu Corporation, Japan) and 2 mL/min of 80% (v/v) acetonitrile at 40 °C. The activity of the enzymes was measured using the glucose residual.

Levan was quantified in terms of its detected molecular weight using high-performance size exclusion chromatography (HPSEC) [37]. The size exclusion column (TSKgel Amide-80, 5 μm, 250 × 4.6 mm) was equipped with an HPLC instrument (Shimazu LC-20A series, Shimazu Corporation, Japan). A 0.1 M NaCl eluent flow rate of 1 mL/min was used at 40 °C. For detection, the RI detector was used. Calibration was performed using standard polyethylene oxide (TSKgel SE-kit, TOSOH Corporation, Japan).

3. Result

3.1. Effect of sucrose concentrations on B. siamensis growth and in transfructosylation and hydrolysis reactions

Sucrose concentration considerably affects *B. siamensis* production, transfructosylation, and hydrolysis catalysis. Figures 1a and 1b illustrate the growth rate of *B. siamensis* in fermented medium containing 20% and

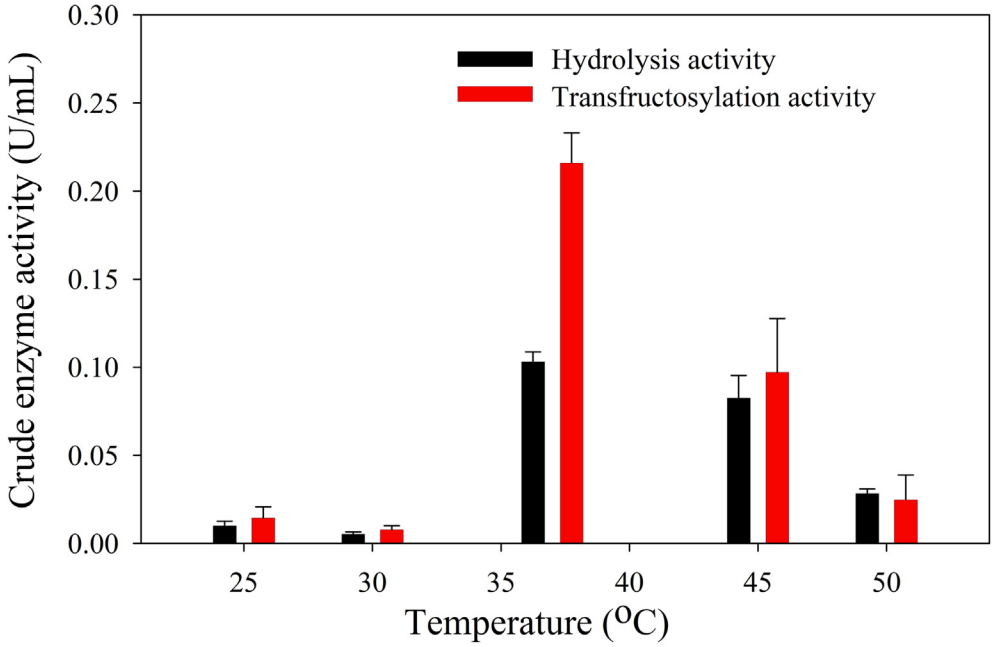


Figure 3. Effects of the initial culture temperature on the crude enzyme activity of *B. siamensis* at 25 °C, 30 °C, 37 °C, 45 °C, and 50 °C with a pH of 6.0 after 12 h.

30% (w/v) sucrose, respectively. The results indicate that *B. siamensis* growth was slightly different from biomass proliferation. At the stationary phase, the OD600 adsorption of biomass was approximately 1.87 and 1.85 in 20% and 30% (w/v) sucrose, respectively. As predicted, biomass proliferation decreased slightly as sucrose concentration increased.

B. siamensis growth was found to be affected by varied sucrose concentrations and sugar reduction in the reaction was observed (Figures 1a and 1b). Sucrose reduction by the levansucrase enzyme resulted in levan production via *B. siamensis* fermentation, followed by an enzymatic reaction with sucrose as the substrate. It was observed that during the early exponential phase of growth, 20% (w/v) sucrose was reduced by 90% until the stationary phase. While in culture containing 30% (w/v) sucrose, the growth decreased with 83.3% biomass reduction. We observed the formation of monosaccharides (glucose and fructose) in the reaction medium. The accumulation of monosaccharides was attributed to sucrose conversion to levan, as evidenced by increased fructose and glucose levels in the medium. The fructose concentration and unidentified fermentation products accumulated in 20% (w/v) sucrose were comparable to those in 30% (w/v) sucrose. In both conditions, glucose was generated at an increasing rate during fermentation.

The effect of sucrose concentration on transfructosylation and hydrolysis activities was investigated (Figures 2a and 2b). Both transfructosylation and hydrolysis activities increased steadily during the 6 h in the culture medium containing 20% (w/v) sucrose, resulted to approximately 0.26 and 0.17 U/mL, respectively. The reduction of transfructosylation and hydrolysis activities occurred after 6–24 h of fermentation. In the case of a culture medium containing 30% (w/v) sucrose, the transfructosylation activity increased during fermentation, and the highest activities appeared in 0.33 U/mL at 24 h. At the same time, the hydrolysis activity increased by approximately 0.20 U/mL at 6 h but dropped to 0.20 U/mL at 24 h. However, both conditions of sucrose concentrations showed that the transfructosylation activity was higher than hydrolysis activity.

3.2. Effect of initial temperature on B. siamensis-catalyzed levansucrase activity

The effect of incubation temperature on the crude levansucrase activity of *B. siamensis* was estimated using free-cell fermentation (Figure 3). The temperature at which the optimal transfructosylation activity was observed is 37 °C, compared to 20 °C, 30 °C, 45 °C, and 50 °C. As the temperature increased from 37 °C to 45 °C, the activity of transfructosylation decreased from 0.22 to 0.10 U/mL. Hydrolysis activity decreased slightly from 0.10 to 0.08 U/mL as the temperature increased from 37 °C to 45 °C. Both transfructosylation and hydrolysis activities were significantly reduced to less than 0.05 U/mL compared to a similar activity value.

3.3. Effect of glucose and fructose concentrations on B. siamensis-catalyzed levansucrase activity

The effects of monosaccharides on crude levansucrase activity were examined using free-cell fermentation with *B. siamensis*. Glucose and fructose were added to a solution containing predominantly 20% (w/v) sucrose and incubated at 37 °C with a pH of 6.0 to assess activity.

When the glucose content was increased from 0% to 10% (w/v) (Figure 4a), the hydrolysis activity decreased from 0.230 to 0.054 U/mL. Transfructosylation activity has also decreased from 0.36 to 0.25 U/mL as the glucose content increased from 0% to 6% (w/v), and a slight increase was observed at higher glucose concentrations. While adding fructose at concentrations ranging from 0% to 10% (w/v) (Figure 4b), hydrolysis and transfructosylation activities substantially decreased. Hydrolysis activity was inhibited at fructose concentrations ranging from 8% to 10% (w/v). While increasing the fructose concentration from 2% to 10% (w/v) the transfructosylation activity increased from 0.05 to 0.20 U/mL. The high fructose content of the medium slightly enhances transfructosylation activity. Thus, increasing the level of fructose or glucose in the medium is inhibitory to the hydrolysis activity, and the addition of these monosaccharides should be carefully monitored to ensure adequate levansucrase secretion and activity.

3.4. Characterization of levansucrase enzyme

We previously confirmed that *B. siamensis* levansucrase is an extracellular enzyme [33]. Figure 5a depicts the result of anion-exchange chromatography of the crude enzyme on a Toyopearl DEAE-650M chromatography column. The anion-exchange elution profile exhibited a broad peak. Extra-levansucrase showed a specific activity of 15.95 U/mg and a recovery rate of 78.75% after being purified 11.5 times (Table 3). Levansucrase activity was observed in NaCl fractions 11–23

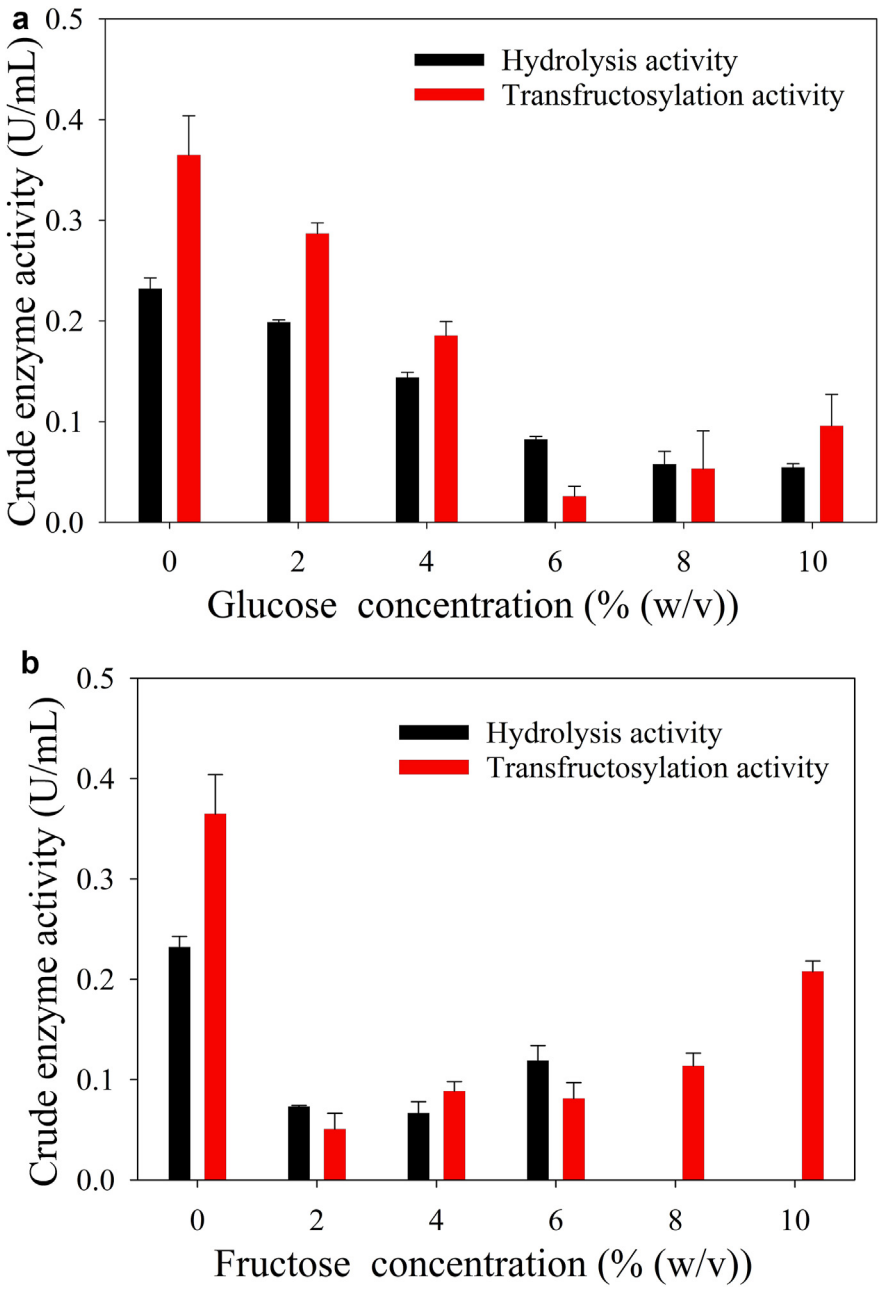


Figure 4. Effects of the secondary carbon source on the crude enzyme activity of *B. siamensis* under control conditions with a pH of 6.0 after 12 h. Additional glucose (a) and fructose (b): 0%, 2%, 4%, 6%, 8%, and 10% (w/v).

when the elution NaCl concentration was between 0.2 and 0.3 M. The protein molecular weight was estimated to be 30 kDa using analytical SDS-PAGE (Figure 5b).

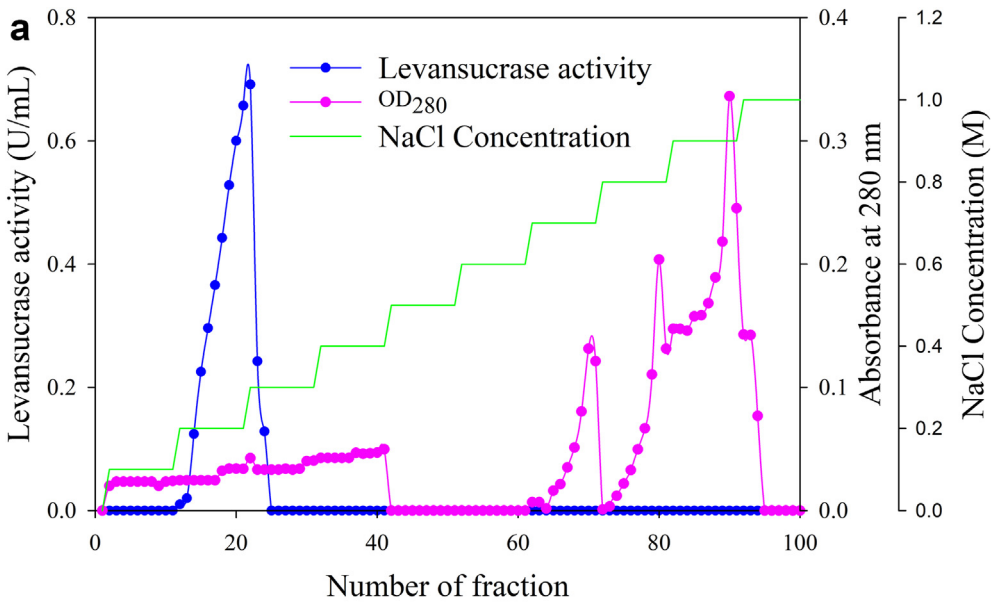
3.5. Effect of sucrose concentrations in levansucrase activity

We optimized the substrate concentration at 37 °C using concentrations of 5%, 10%, and 20% (w/v) sucrose in 50 mM phosphate buffer to study the influence of sucrose concentration on levansucrase activity and levan synthesis (pH 6.0). Sucrose concentration and reaction times were found to be the primary determinants of levansucrase activity (Figure 6). The hydrolysis activity was greater than the transfructosylation activity throughout 3 h under the three conditions because sucrose was catalyzed to release glucose and fructose. The transfructosylation activity was activated and subsequently increased after 3 h. The increase of sucrose results from the fructose concentration increasing to a level that allows it to be incorporated into acceptor products such as levan [41].

Transfructosylation and hydrolysis activities were approximately 0.18 and 0.09 U/mL in 5% (w/v) sucrose after 3 h. The ratio of transfructosylation to sucrose hydrolysis was approximately 2.0 (Figure 6a). After 3 h, the transfructosylation activity was 0.20 U/mL, and the hydrolysis activity was 0.15 U/mL in the case of 10% (w/v) sucrose after 3 h (Figure 6b). Similarly, after 12 h, the transfructosylation and hydrolysis activities at a sucrose concentration of 20% (w/v) were approximately 0.26 and 0.12 U/mL. The ratio of transfructosylation to sucrose hydrolysis was approximately 2.20 (Figure 6c).

3.6. Effect of pH conditions in levansucrase activity

Since enzyme configuration and activities are pH-sensitive, the condition of the pH value is critical for enzyme performance [29]. When pH



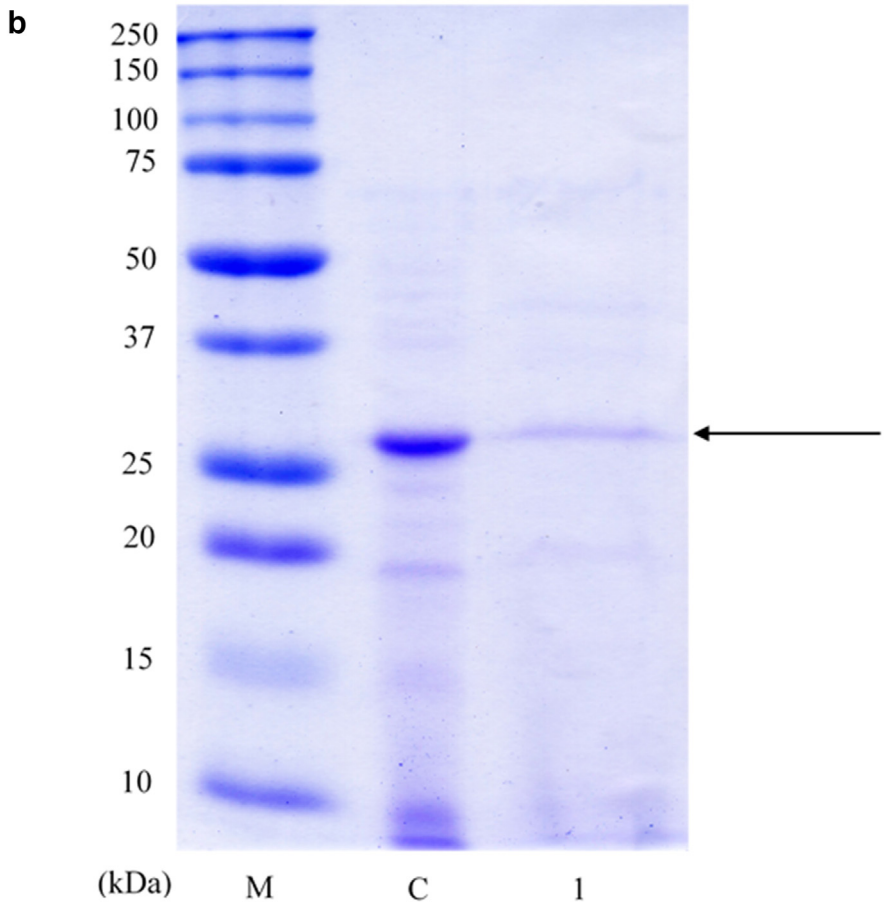


Figure 5. Elution profiles of extracellular levansucrase from *B. siamensis*. Ion exchange chromatography on a Toyopearl DEAE-650M chromatography column using a phosphate buffer, pH 6.0 with crude enzyme preparation. Elution was performed with a concentration of 0–1 M NaCl (5 mL/fraction). The absorption at OD280 and levansucrase activity of each fraction were determined (a). SDS-PAGE analysis of *B. siamensis* extracellular levansucrase. Lane 1, molecular mass markers. Lane 2, eluted proteins with levansucrase activity purified using a Toyopearl DEAE-650M column at 0.8 M NaCl. Lane M indicates the molecular weight of the protein marker. Lane C indicates crude enzyme. Lane 1 indicates elution with 0.3 M NaCl. The arrow indicates the position of levansucrase (b).

values are altered from optimal values, enzyme activity decreases [28]. Thus, we investigated the effect of pH on levansucrase activity and levan production (Figure 7). The results indicated that the most significant differences occurred in phosphate buffers with pH values ranging from 6.0 to 8.0. Levansucrase activity was maintained at pH 6.0 and significantly decreased at pH greater than 7.0. Levansucrase activity was maintained at pH 6.0 in the acetate buffer but decreased to 48% relative activity at pH 5.0. The acidic solution with a pH of 4.0 had a significant effect on the levansucrase activity.

3.7. Effect of varying temperatures on levansucrase activity

The effect of temperature changes on levansucrase activity was studied (Figure 8). At 25 °C and 37 °C, the transfructosylation activity

Table 3. Purification of extracellular levansucrase by culturing *B. siamensis* under optimum condition.

Protein purification	Enzyme activity (U/mL)	Protein concentration (mg/mL)	Specific enzyme activity (U/mg)	Recovery (%)	Protein purify (fold)
Crude enzyme	320	230	1.39	100	1
Toyopearl DEAE-650M	252	15.8	15.95	78.75	11.47

was higher than the hydrolysis reaction as shown in Figures 8a and 8b, respectively. On the contrary, at 50 °C, the transfructosylation activity became less than the hydrolysis reaction (Figure 8c). As a result, increased transfructosylation activity makes it easier to make levan from sucrose. After 3 h of incubation at 37 °C, the highest transfructosylation activity, 0.24 U/mL, was recorded, as was the maximum levan production. The ratio of transfructosylation to sucrose hydrolysis activity was approximately 1.5:1 after 24 h of incubation at 37 °C.

3.8. Effect of sucrose concentrations, pH, and temperatures on the molecular weight of synthesized levan by purified levansucrase

The effect of physicochemical conditions on levan biosynthesis including sucrose concentration, pH-driven formation, and temperature, on the molecular weight distribution of levan, was investigated (Figure 9). The molecular weight of levan was determined by incubating purified levansucrase with sucrose concentration under fixed conditions. Levan has two peaks on the chromatogram as Figure 9a, one with low molecular weight and the other with a high molecular weight. The retention time for low molecular weight levan was substantially longer than for high molecular weight levan. The high molecular weight was approximately 10^6 kDa, while the low molecular weight was approximately 50 kDa.

The effect of sucrose concentration on levan biosynthesis was investigated (Figure 9b). The results indicated that sucrose concentration had a significant effect on levan biosynthesis. The relationship between sucrose concentration and biosynthesized levan was estimated at 37 °C and 50 mM phosphate buffer (pH 6.0) for 24 h. The highest levan biosynthesis was observed at a 20% (w/v) sucrose medium for 24 h, yielded 14.5% (w/v) of 50 kDa levan and 0.79% (w/v) of 10^6 kDa levan. After 24 h of incubation in a medium containing 10% (w/v) sucrose, 4.01% (w/v) of 50 kDa levan and 2.24% (w/v) of 10^6 kDa levan were produced. However, the lowest levan biosynthesis was observed under medium containing 5% (w/v) sucrose incubated for 24 h yielding 0.18% (w/v) 50 kDa levan and 2.40% (w/v) 10^6 kDa levan.

The effect of pH on the molecular weight distribution of levan was examined (Figures 9c and 9d). The low molecular weight of levan was produced at a concentration of approximately 14.59% (w/v) in 50 mM phosphate buffer (pH 6.0), which was slightly higher than the 50 mM acetate buffer (pH 6.0).

Furthermore, it was discovered that temperature affected levan biosynthesis (Figure 9e). Levan yielded a maximum of 14.59% (w/v) at 37 °C and decreased with increasing or decreasing temperature. It

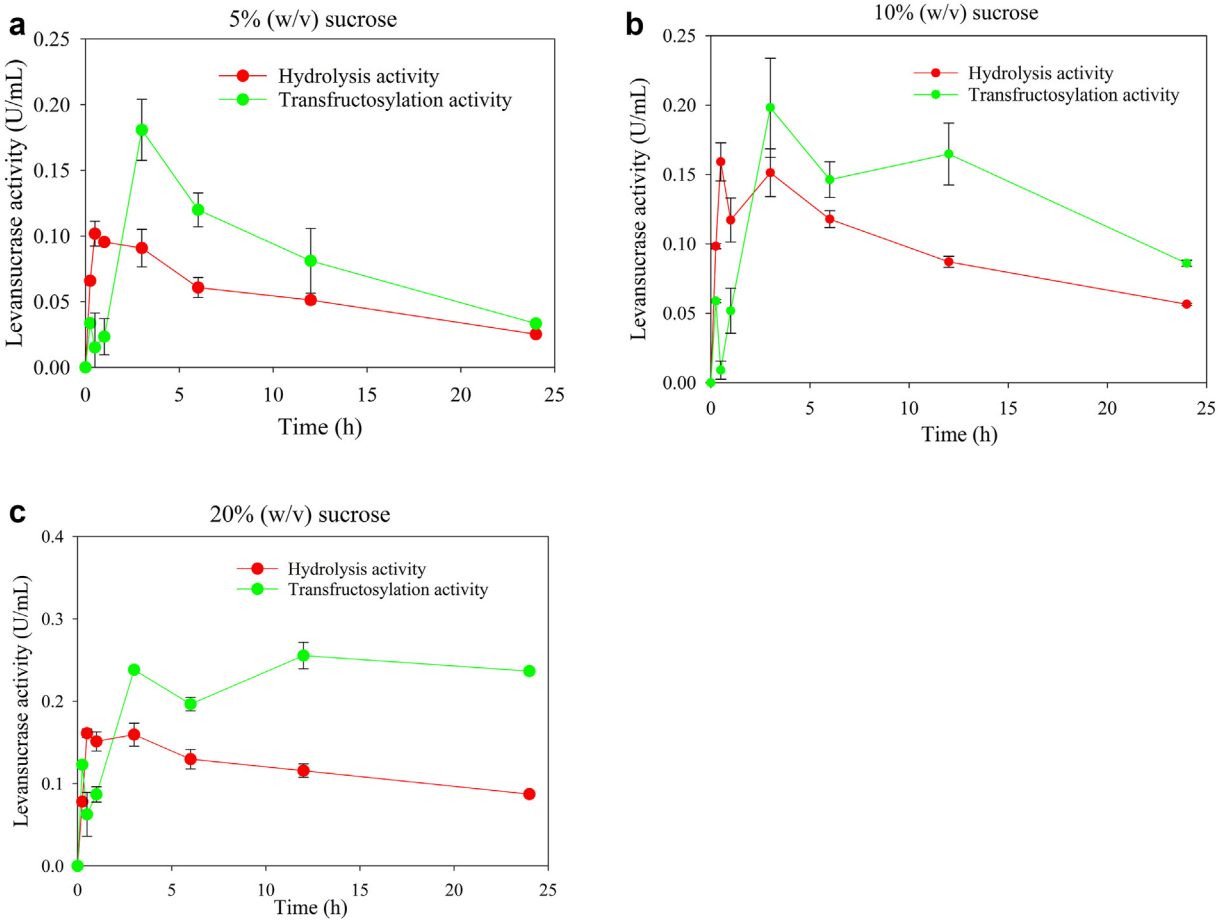


Figure 6. Effects of sucrose concentration on levansucrase activity at 5% (w/v) sucrose (a), 10% (w/v) sucrose (b), and 20% (w/v) sucrose (c) in 50-mM phosphate buffer (pH 6.0) at 37 °C.

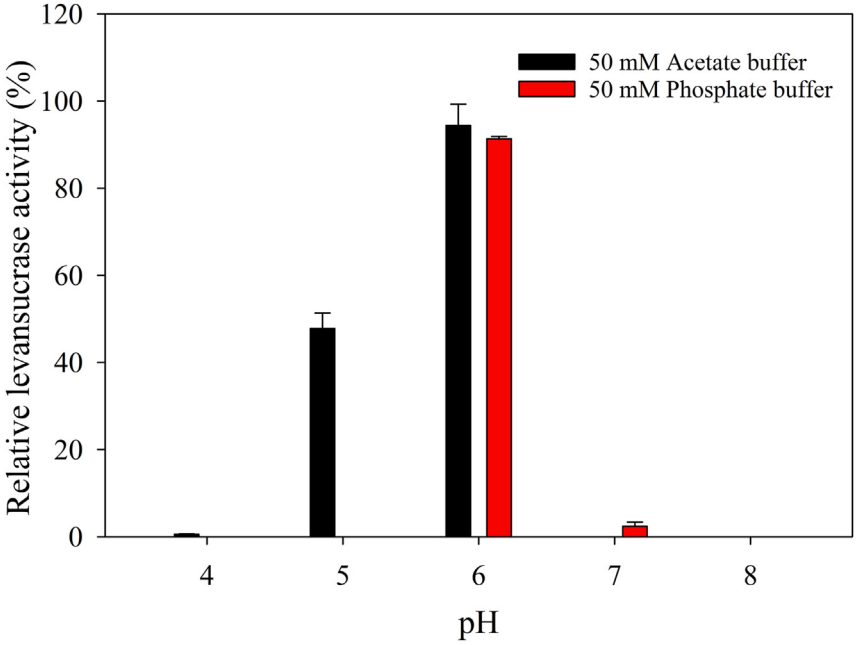


Figure 7. Effects of the initial pH on levansucrase activity in acetate buffer (pH 4.0–6.0) and phosphate buffer (pH 6.0–8.0) at 37 °C after 3 h.

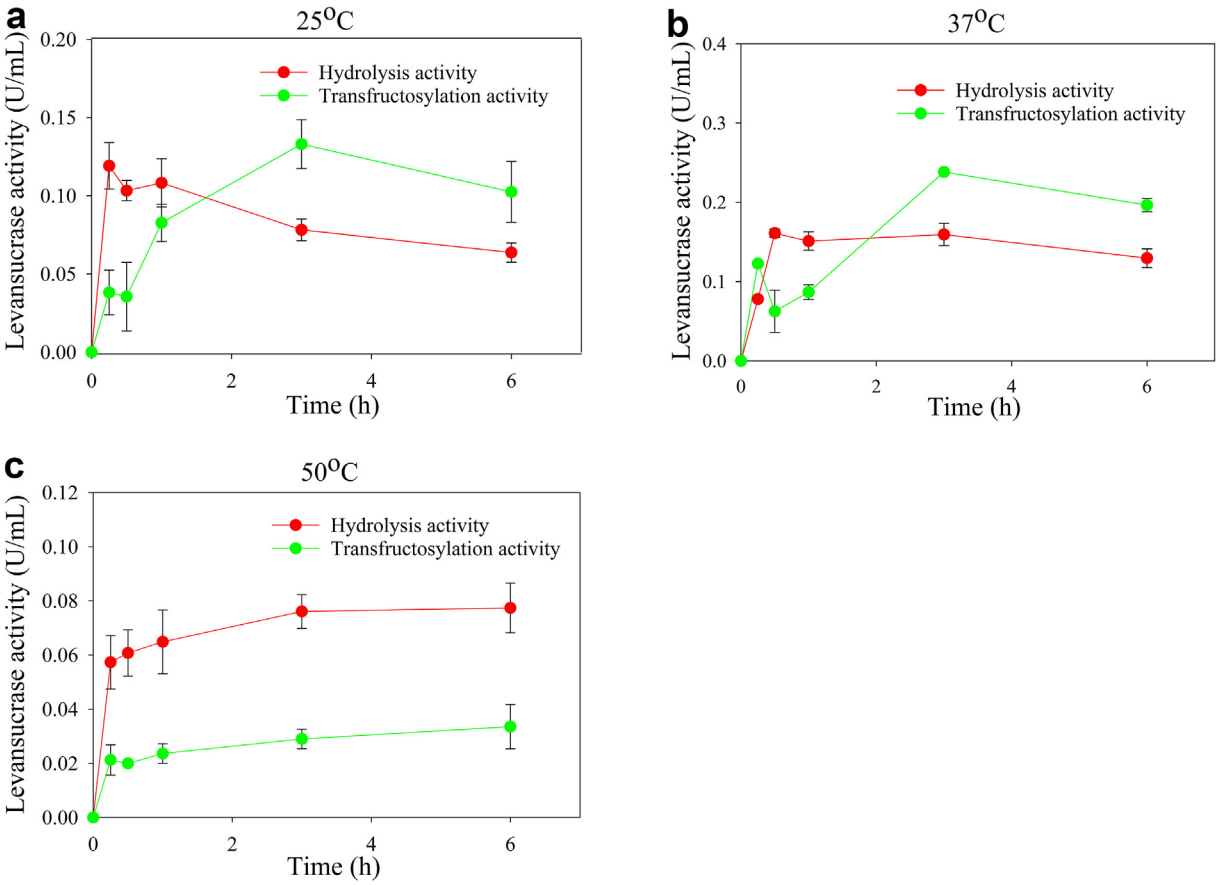


Figure 8. Effects of the initial temperature on enzyme activity under control conditions, including 20% (w/v) sucrose in 50 mM phosphate buffer (pH 6.0) at 25 °C (a), 37 °C (b), and 50 °C (c).

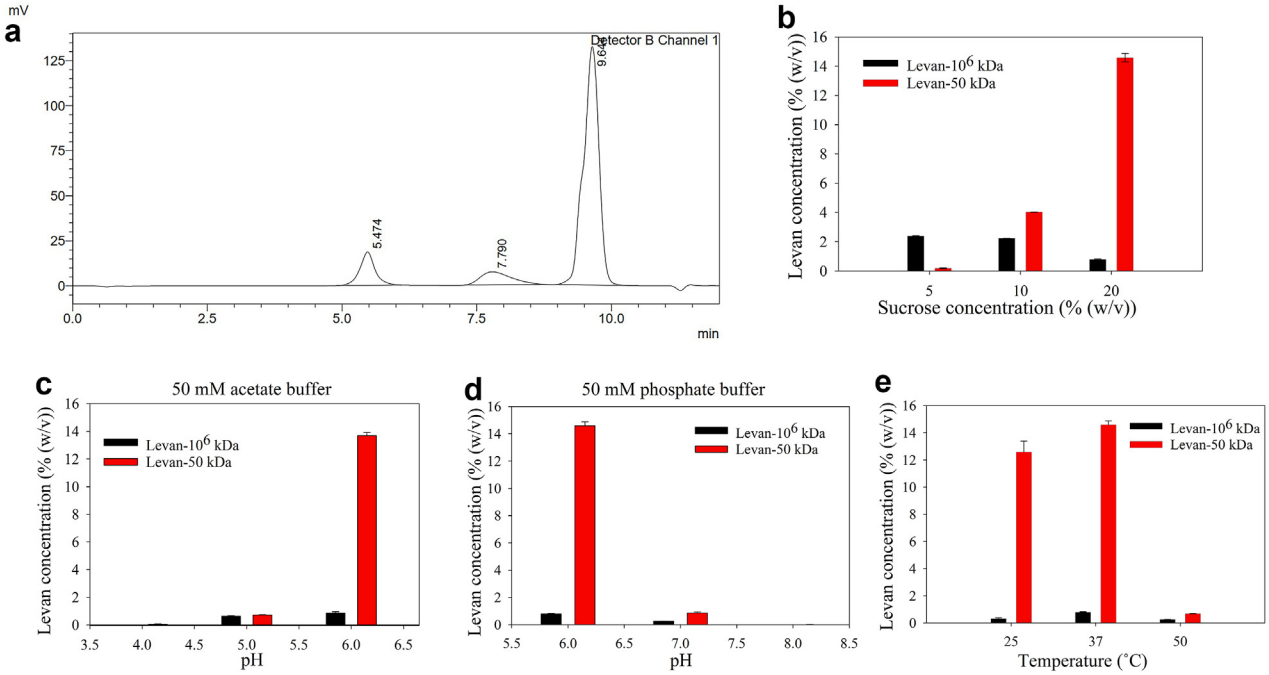


Figure 9. GPC measuring the levan products biosynthesized by *B. siamensis* in a culture medium containing 20% (w/v) sucrose. (a) GPC chromatogram of levan containing high molecular, low molecular, and mobile phase peak (from left to right). (b) Effect of sucrose concentration conditions on the biosynthesis of levan. (c) Effect of the initial pH conditions on the biosynthesis of levan in acetate buffer (pH 4.0–6.0). (d) Effect of the initial pH conditions on the biosynthesis of levan in phosphate buffer (pH 6.0–8.0), and (e) Effect of the initial temperature conditions on the biosynthesis of levan.

decreased slightly to approximately 12.57% (w/v) at 25 °C and significantly decreased to approximately 0.68% (w/v) at 50 °C.

3.9. Kinetics of the levansucrase-catalyzed reaction

The optimal conditions for the production of levansucrase-catalyzed transfructosylation products (including sucrose concentration, pH, and temperature) for modulating their spectrum profile were investigated (Figure 10).

The result showed that catalyzed levansucrase conversion of substrates exhibited distinct reaction time–course profiles. During the first six hours of the reaction, the rate of sucrose conversion remained constant, as did the rate of glucose synthesis owing to hydrolyzed sucrose. During that period, the conversion rate of levan-50 kDa increased rapidly from 7.02% to 28.00%. Since transfructosylation occurs, when the residual fructose is transferred to the molecule of sucrose as acceptor typically via a β-(2–6)-glycosidic linkage. After this reaction time, sucrose was slowly transferred at a lower rate, yielding an 85.57% conversion

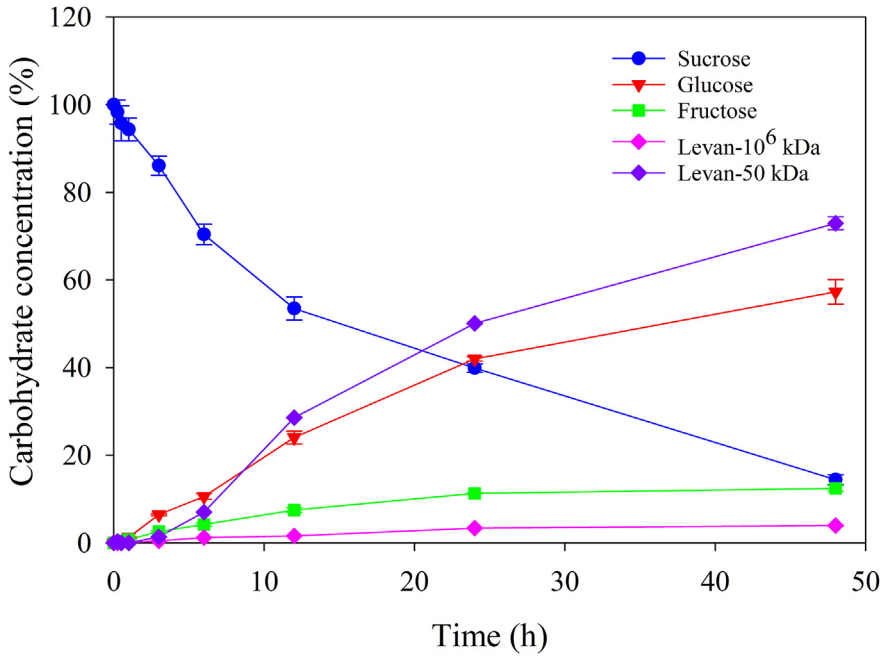


Figure 10. Levan production from sucrose by the purified *B. siamensis* levansucrase. The enzymatic reaction was performed using purified levansucrase with 20% (w/v) sucrose in phosphate buffer with a pH of 6.0 at 37 °C.

Table 4. Purification methods and corresponding molecular weight of levansucrase produced by *Bacillus* strains.

Bacterial strain	Purification method	Condition	MW (kDa)	Ref.
B. siamensis	Anion-exchange chromatography	0–1 M NaCl concentration (elution solution)	30	this study
B. subtilis DSM 347	Anion-exchange chromatography	160, 400, 800, and 2000 mM Sorensen's phosphate buffers (pH 6.0) (elution solution)	51	[45]
B. subtilis NRC16	The acetone fractionations	30%–90% final acetone concentrations	14	[44]
B. licheniformis	Anion-exchange chromatography	0–1 mM NaCl concentration (elution solution)	45	[46]
	Gel filtration chromatography	20 mM phosphate buffer (pH 6.5) and a flow rate of 30 mL/h		
B. licheniformis 8-37-0-1 (recombinant)	Anion-exchange chromatography	0–0.4 M NaCl concentration (elution solution)	51	[54]
B. licheniformis RN-01	Anion-exchange chromatography	0–1 M NaCl concentration (elution solution)	52	[47]
	Butyl-Toyopearl-650 M column	0–1.33 M of ammonium sulfate concentration (elution solution)		
B. methylotrophicus SK 21.002	Anion-exchange chromatography	0–1 mM NaCl concentration at the flow rate of 1 mL/min	60	[48]
	Gel filtration chromatography	20 mM sodium phosphate buffer (20 mM, pH 6.5) and a flow rate of 0.5 mL/min		
B. amyloliquefaciens	PEG 200 fractionation	30% (v/v) concentration	59	[41]
	Gel filtration chromatography	50 mM potassium phosphate buffer (pH 6.0) containing 0.15 M NaCl		

Table 5. Comparison of optimum conditions for levan produced by various levansucrases.

Microbial source	Levansucrase	Sucrose (% (w/v))	Optimum condition	Yield (%)	Ref.
B. siamensis	Purified	20	37 °C, pH 6.0, 48 h	72.9	This study
B. methylotrophicus SK 21.002	Purified	30	37 °C, pH 6.0, 16 h	33.0	[50]
B. goodwinii	Purified Recombinant	50	35 °C, pH 6.0, 6 h	37.0	[16]
L. reuteri LTH5448.	Purified	50	35 °C, pH 6.0, 12 h	36.6	[51]
	Purified	36	50 °C, pH 6.0, 24 h	55.6	[31]

yield after 48 h. A decrease in sucrose content and/or product inhibition resulted in a reduced conversion rate. In the case of levan-50 kDa conversion, it was expanded continuously to achieve a 72.94% conversion. Furthermore, after 48 h of reaction, levansucrase from *B. siamensis* produced levan with a molecular weight of 10^6 kDa that was converted at a rate of 3.95%.

4. Discussion

With an increasing interest in levan for its possible health advantages related to its prebiotic, biological, and functional properties, researchers are focused on developing efficient production methods using highly biocatalytic enzymes. *B. siamensis* was previously demonstrated to synthesize the novel extracellular levansucrase capable of converting sucrose to levan [33]. The crude enzyme biocatalytic properties were also investigated to determine the enzyme activity.

With the aim to reduce production costs and efficiently produce levan, the present study optimized culture conditions for *B. siamensis* and determined the amount of levansucrase produced, enzyme activities, and levan biosynthesis. The results confirm that a high sucrose concentration of 20% (w/v) to 30% (w/v) had a marginal effect on microbial biomass. At low sucrose concentrations, such as less than 10% (w/v), bacteria consumed a significant percentage of the carbon source for cell growth, according to Belghith et al. [34]. In contrast, high sucrose concentrations inhibited cell growth but increasingly induced enzyme secretion. However, sucrose concentration had a significant effect on increasing transfructosylation activity rather than hydrolysis activity. The relationship between crude levansucrase of *B. siamensis* and sucrose concentration was examined. Sucrose increased enzyme excretion and favored transfructosylation reactions. To compensate for the increased osmotic stress, cells secrete a transfructosylating enzyme that converts fructose to sucrose resulting in the formation of levan. According to Zeng et al. [42], the biotransformation utilizing free-cell biocatalysts can be classified into two steps: sucrose hydrolysis and fructose transfer via the transfructosylation reaction. At low sucrose concentrations, the hydrolysis activity was relatively high, while the transfructosylation reaction was relatively slow, resulting in many free glucose and fructose residues. Furthermore, the content of monosaccharides such as glucose and fructose had a significant effect on reducing hydrolysis and transfructosylation activities, as Ananthalakshmy and Gunasekaran reported [43]. These findings revealed that cells under various conditions exhibited a variety of enzyme reaction mechanisms. Thus, the enzyme activity should be elucidated in stages using purified levansucrase.

Toyopearl DEAE-650M ion-exchange chromatography column and SDS-PAGE analysis were utilized to investigate the enzyme purity and enzymatic characteristics of *B. siamensis* levansucrase (Figure 5). Numerous reports indicate that the molecular weights of levansucrase enzymes produced from *Bacillus* strains vary and the enzyme properties are strain-specific (Table 4). The molecular weights of enzymes from *B. subtilis* species vary considerably, ranging from 14 kDa for levansucrase from *B. subtilis* NRC16 to 51 kDa for levansucrase from *B. subtilis* DSM 347 [44,45]. *Bacillus licheniformis* strains can synthesize molecules with similar molecular weights, ranging from 45 to 52 kDa [46, 47]. While *B. methylotrophicus* species synthesized a 60 kDa protein with a molecular weight similar to that of *B. amyloliquefaciens* levansucrase [48].

For optimal levansucrase activity in the biosynthesis of levan, several factors have been identified that influence the levansucrase mechanisms and the molecular weight distribution of levan, including sucrose concentration, and pH. The properties of *B. siamensis* levansucrase were compared to those of levansucrase from previous studies, as shown in Table 5.

The current study demonstrates that as sucrose concentrations increase, transfructosylation activity also increases. It is in accordance with numerous findings on levansucrase activity [14, 16, 49]. In addition, sucrose concentrations affect the levan molecular weight distribution. A higher amount of smaller molecular weight levan is produced in the higher sucrose concentration because sucrose is an acceptor of the fructosyl-enzyme intermediate to induce the synthesis of short-chain oligosaccharides. On the other hand, the capacity to accept fructosyl

moieties of the fructosyl-enzyme intermediate decrease at lower sucrose concentration. Similar results have been reported by Porras-Dominguez et al. [30] and Wu Fang-Chen et al. [49]. Therefore, sucrose concentration is a key factor in levan biosynthesis because a suitable sucrose concentration enhances the transfructosylation process and levan molecular weight formation.

Numerous reports also demonstrated that pH affects enzyme performance and levan biosynthesis. According to Goldman et al. [28], levansucrase may be synthesized in two active forms, depending on the pH and ionic strength. The ability of soluble levansucrase to synthesize fructooligosaccharide is inhibited by the formation of insoluble but active protein microfibrils in response to changes in pH. At pH less than 6.0, the enzyme catalyzes almost exclusively the synthesis of low molecular weight levan in its microfibril form. Additionally, studies on *Brenneria goodwinii* [16], *Bacillus subtilis* [38, 45], *Bacillus subtilis* NATTO [27], *Bacillus methylotrophicus* [50], *Klebsiella* species [20], *Lactobacillus reuteri* [51], and *Zymomonas mobilis* [14] have demonstrated that a pH range of 5.0–6.5 is optimal for levansucrase activity. However, pH extremes have a significant negative effect on levansucrase activity.

The levansucrase enzyme revealed an interesting range of optimal temperatures for various activities and levan biosynthesis. At 37 °C, *Bacillus methylotrophicus* SK 21.002 had the maximum levansucrase activity, according to Tao Zhang et al. [50]. At 50 °C, it was determined that elevated temperatures have a significant negative effect on levansucrase activity via the hydrolysis reaction, which is greater than transfructosylation activity. The greatest hydrolytic activity of *Pseudomonas syringae* levansucrase was previously demonstrated in the study of Ursula Hettwer et al. [52] at 60 °C.

The production of levansucrase-catalyzed transfructosylation products demonstrates that levansucrase from *B. siamensis* biosynthesis has a broad molecular weight distribution (50–10^6 kDa) affected by the reaction conditions. Levansucrase from *B. siamensis* is capable of synthesizing levan at the desired molecular weight for its application. In medical applications, low molecular weight levan was used to promote antioxidant and antiinflammatory activity. Levansucrase generated from *B. subtilis* NRC1aza produced two unique kinds of levan with molecular weights of 85.23 kDa and 31.95 kDa [11]. When coupled with DPPH, they showed considerable free radical scavenging action. The low molecular weight of levan (about 37.62 kDa) reduced lipid peroxidation in a flaxseed oil emulsion following Fe^{2+} inductor treatment [53]. As a result of the vast spectrum of levan molecular weights, levan with specialized molecular weights for specific applications are being developed. Given the versatility of levansucrase produced by *B. siamensis* as demonstrated in the current study strongly suggests that the bacterial species can be of promising candidate in mass production of levan and as producer of enzyme important in the various industrial processes.

Declarations

Author contribution statement

Pongtorn Phengnoi, Nattapong Thakham and Witawat Jangiam: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Tanawat Rachphirom: Performed the experiments; Analyzed and interpreted the data.

Nuttinee Teerakulkittipong and Gary Antonio Lirio: Contributed reagents, materials, analysis tools or data; Analyzed and interpreted the data.

Funding statement

Assoc. Prof. Dr. Witawat Jangiam was supported by National Research Council of Thailand [23/2562]. Pongtorn Phengnoi was supported by Thailand Research Fund Grant for new researcher [M001/2562].

Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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