Synthesis of raffinose by transfructosylation using recombinant levansucrase from Clostridium arbusti SL206

Wenjing Li, a Shuhuai Yu, a Tao Zhang, a Bo Jiang a, b and Wanmeng Mu a, b*

Abstract

BACKGROUND: Raffinose, a functional trisaccharide of α-D-galactopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside, is a prebiotic that shows promise for use as a food ingredient.

RESULTS: In this study, the production of raffinose from melibiose and sucrose was studied using whole recombinant Escherichia coli cells harboring the levansucrase from Clostridium arbusti SL206. The reaction conditions were optimized for raffinose synthesis. The optimal pH, temperature and washed cell concentration were pH 6.5 (sodium phosphate buffer, 50 mmol L⁻¹), 55 °C and 3% (w/v), respectively. High substrate concentrations, which led to low water activity and thus reduced levansucrase hydrolysis activity, strongly favored the production of raffinose through the fructosyl transfer reaction. Additionally, high concentrations of excess acceptor and donor glycosides favored raffinose production. When 30% (w/v) sucrose and 30% (w/v) melibiose were catalyzed using 3% (w/v) whole cells at pH 6.5 (sodium phosphate buffer, 50 mmol L⁻¹) and 55 °C, the highest raffinose yield was 222 g L⁻¹ after a 6 h reaction. The conversion ratio from each substrate to raffinose was 50%.

CONCLUSION: Raffinose could be effectively produced with melibiose as an acceptor and with sucrose as a fructosyl donor by whole recombinant E. coli cells harboring C. arbusti levansucrase. The yield from E. coli was significantly higher than those of the previously reported Bacillus subtilis levansucrase and fungal α-galactosidases.

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Keywords: synthesis; Clostridium arbusti SL206; levansucrase; raffinose; transfructosylation
Levansucrase (EC 2.4.1.10), which catalyzes the formation of levans from sucrose, is found in a wide range of microorganisms. Levansucrase is a naturally occurring polysaccharide of a β-2,6-linked D-fructose homopolymer and is generally produced as a byproduct of levansucrase activity formed during microbial sucrose fermentation. In addition to levansucrase biosynthesis, levansucrase simultaneously produces levan-type fructooligosaccharides (FOS) and catalyzes sucrose hydrolysis. Levansucrase, with sucrose as the transfructosyl donor, also catalyzes other transfructosylation reactions and produces a series of sucrose analogs from various transfructosyl acceptor substrates, including maltose, lactose, cellobiose, melibiose, isomaltose, D-galactose, D-fructose and D-xyllose.

In this work, a recombinant levansucrase from Clostridium arbusti SL206 was cloned and expressed in Escherichia coli. The recombinant E. coli strain was cultivated in Luria–Bertani (LB) medium consisting of 10 g L\(^{-1}\) tryptone, 5 g L\(^{-1}\) yeast extract and 10 g L\(^{-1}\) sodium chloride at pH 7.0 with 50 μg mL\(^{-1}\) ampicillin in a rotary shaker at 37°C and 200 rpm. The optical cell density at 600 nm (OD\(_{600}\)) reached 0.6–0.8, IPTG was added to a final concentration of 1 mmol L\(^{-1}\) to induce the expression of levansucrase at 20°C for 24 h. The cells were then harvested by centrifugation (10,000 × g, 10 min, 4°C) and washed twice with sodium phosphate buffer (50 mmol L\(^{-1}\), pH 7.0). The cell pellets were weighed and used as a levansucrase source for the enzymatic reactions. Levansucrase activity was 0.21 U mg\(^{-1}\) wet cells.

Levansucrase activity assay
Enzyme activity was analyzed by measuring the amount of D-glucose released following sucrose hydrolysis. The carbohydrates were measured by HPLC. One unit of levansucrase activity is defined as the amount of cells producing 1 μmol D-glucose from 5% (w/v) sucrose min\(^{-1}\) at 55°C and pH 6.5 (sodium phosphate buffer, 50 mmol L\(^{-1}\)). Statistical data were obtained based on the three replicates.

Quantitative measurement of carbohydrates
Quantifications of D-fructose, D-glucose, sucrose, melibiose and raffinose were performed via an HPLC system (Agilent 1260, CA, USA) equipped with a refractive index detector (RID) and an Asahipak NH2P-50-4E column (Shodex, Tokyo, Japan) at 30°C.

**MATERIALS AND METHODS**

**Chemicals and reagents**
Standard raffinose and melibiose were purchased from Sigma (St Louis, MO, USA) for high-performance liquid chromatography (HPLC) analysis. The HPLC-grade acetonitrile was obtained from Tedia Co. Inc. (Fairfield, OH, USA). Yeast extract and tryptone were from Oxoid Ltd (Basingstoke, UK). Isopropyl-β-D-1-thiogalactopyranoside (IPTG) and ampicillin were from Sangon Biotech Co. Ltd (Shanghai, China). Other chemicals were of analytical grade and were mainly purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). The ultrapure water was used for HPLC fluid phase, and other experimental samples were prepared with deionized water.

**Heterologous expression of C. arbusti levansucrase in E. coli**
The complete genome of Clostridium arbusti SL206 was recently submitted to GenBank (NCBI accession number: NZ_BAEV01000048). The genome sequence contains a putative levansucrase gene (gene locus_tag: RH99_RS10050; protein ID: WP_010237336.1). The full-length nucleotide sequence of the putative levansucrase-encoding gene was synthesized by Shanghai General Biotech Co. Ltd (Shanghai, China) and cloned into the expression vector pET-22b(+) with Ndel and Xhol restriction sites, generating the recombinant plasmid. The recombinant plasmid was then transformed into host E. coli BL21(DE3) for heterologous expression of the recombinant levansucrase.

The transformed E. coli strain was cultivated in Luria–Bertani (LB) medium consisting of 10 gL\(^{-1}\) tryptone, 5 gL\(^{-1}\) yeast extract and 10 gL\(^{-1}\) sodium chloride at pH 7.0 with 50 μg mL\(^{-1}\) ampicillin in a rotary shaker at 37°C and 200 rpm. When the optical cell density at 600 nm (OD\(_{600}\)) reached 0.6–0.8, IPTG was added to a final concentration of 1 mmol L\(^{-1}\) to induce the expression of levansucrase at 20°C for 24 h. The cells were then harvested by centrifugation (10,000 × g, 10 min, 4°C) and washed twice with sodium phosphate buffer (50 mmol L\(^{-1}\), pH 7.0). The cell pellets were weighed and used as a levansucrase source for the enzymatic reactions. Levansucrase activity was 0.21 U mg\(^{-1}\) wet cells.

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Quantifications of D-fructose, D-glucose, sucrose, melibiose and raffinose were performed using an HPLC system (Agilent 1260, CA, USA) equipped with a refractive index detector (RID) and an Asahipak NH2P-50-4E column (Shodex, Tokyo, Japan) at 30°C.
with 75% (v/v) acetonitrile as the mobile phase at a flow rate of 1 mL min⁻¹. Three replicates were implemented to ensure the precision of all data.

**Identification of transfructosylation product from melibiose**

Following the enzymatic reaction of sucrose and melibiose with the recombinant *E. coli* harboring levansucrase activity, the reaction product was fractionated using an HPLC system (Waters Corporation, MA, USA) equipped with an RID and a preparation column (XBridge™ Prep Amide; 5 μm, 10 mm ID × 250 mm, Waters). The elution was performed at room temperature with 75% (v/v) acetonitrile as the mobile phase at a flow rate of 2.5 mL min⁻¹. The sugar solution collected was concentrated and freeze-dried in a 4.5 L FreeZone freeze-dry system (Labconco Corp, MO, USA) overnight. The freeze-dried powder was dissolved in D₂O and subjected to NMR measurement experiments. To identify the product, ¹H and ¹³C NMR spectra were recorded with a Bruker Avance III 400 MHz digital NMR spectrometer (Bruker, Karlsruhe, Germany).

**Effects of pH and temperature on raffinose synthesis**

Three buffer systems (50 mmol L⁻¹, sodium acetate buffer (pH 4.0–5.5), sodium phosphate buffer (pH 6.0–7.5) and Tris–HCl buffer (pH 8.0–9.0), were used for studying the effect of pH on raffinose synthesis at 55 °C. The effect of temperature was investigated in sodium phosphate buffer (50 mmol L⁻¹, pH 6.5) by measuring the amount of raffinose produced while varying the temperature from 20 to 80 °C. All reactions were performed using 3% (w/v) washed whole cells in a reaction mixture (1 mL) containing 24% (w/v) sucrose and 24% (w/v) melibiose.

**Effect of cell amount on raffinose synthesis**

The reactions were performed in sodium phosphate buffer (50 mmol L⁻¹, pH 6.5) at 55 °C for 6 h, in a 1 mL reaction mixture containing 24% (w/v) sucrose and 24% (w/v) melibiose. The amount of wet cells added varied from 1% to 14% (w/v) to study the effect of cell amount on raffinose synthesis.

**Effect of substrate concentration on raffinose synthesis**

Ten different concentrations (3%, 6%, 9%, 12%, 15%, 18%, 21%, 24%, 27% and 30%, w/v) of sucrose and melibiose (equal concentrations) were used to measure the synthesis of raffinose. All reactions were performed at pH 6.5 (sodium phosphate buffer, 50 mmol L⁻¹) and 55 °C using 3% (w/v) washed whole cells.

**Effect of substrate ratio on raffinose synthesis**

To investigate the effect of the sucrose:melibiose ratio on raffinose synthesis, the ratios (sucrose (w/v):melibiose (w/v)) were set to 18:30%, 24:30%, 30:30%, 36:30%, 30:36%, 30:24% and 30:18%. All reactions were performed at pH 6.5 (sodium phosphate buffer, 50 mmol L⁻¹) and 55 °C using 3% (w/v) washed whole cells for 6 h in 1 mL reaction mixture.

**Raffinose synthesis from sucrose and melibiose**

The synthesis of raffinose from sucrose and melibiose was analyzed in 100 mL of reaction mixture containing 30% (w/v) sucrose and 30% (w/v) melibiose. The reaction was performed at pH 6.5 (sodium phosphate buffer, 50 mmol L⁻¹) and 55 °C using 3% (w/v) washed whole cells. The changing concentrations of the sugars (D-fructose, D-glucose, sucrose, melibiose and raffinose) were analyzed by HPLC as described above.

**RESULTS AND DISCUSSION**

**Heterologous expression of *C. arbusti* levansucrase**

To date, levansucrases from many microorganisms have been identified. However, to the best of the authors’ knowledge, levansucrases from the *Clostridium* species have not yet been characterized. The whole genome sequence of *C. arbusti* SL206 was recently submitted to GenBank with accession number NZ_BAEV01000048. In the present work, the putative levansucrase-encoding gene from *C. arbusti* SL206 was cloned into the pET-22b (+) expression plasmid containing the inducible T7 lac promoter. The recombinant plasmid was transformed into *E. coli* BL21(DE3), and the recombinant levansucrase was efficiently expressed by IPTG induction (data not shown). After heterologous expression by IPTG induction, the recombinant *E. coli* clearly exhibited sucrose hydrolysis and transfructosylation activity compared to non-induced cells.

**Determination of transfructosyl oligosaccharide production from sucrose and melibiose**

Using sucrose and melibiose as substrates, the recombinant *E. coli* harboring *C. arbusti* levansucrase produced an oligosaccharide as the main product in addition to D-glucose and D-fructose;
Fructose production was significantly lower than D-glucose production (Fig. 2), indicating that the transfructosylation reaction occurred.

To identify the transfructosyl oligosaccharide produced, 13C (supporting information, Fig. S1) and 1H NMR spectra (Fig. S2) of the reaction product were measured. The chemical shifts of 13C NMR spectra for the produced compound are listed in Table 1. The chemical shifts were compared to previously reported results of raffinose production30,31. The 13C NMR spectrum revealed 18 carbons with three anomeric carbons (δC, 103.765, 98.454, and 92.075 ppm) (Fig. S1). Two protons on anomeric sugar carbons (δH, 5.409 and 4.976 ppm) were identified from the 1H NMR spectrum (Fig. S2). Despite a small difference in chemical shift between reaction product and the reported raffinose,30 which can be ascribed to the different calibration (external TMS in Park et al.29 and internal DSS in this work), the chemical shift showed high similarity. In addition, the 13C and 1H NMR analyses of standard raffinose were performed (Fig. S1) and showed the same spectra as those of the compound produced by recombinant C. arbusti levansucrase from sucrose and melibiose. The transfructosyl oligosaccharide produced by levansucrase from melibiose was identified as raffinose [α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside].

**Table 1. Chemical shifts in 13C spectra of raffinose produced from sucrose and melibiose**

<table>
<thead>
<tr>
<th>Group</th>
<th>Carbon atoms</th>
<th>δc of sample</th>
<th>δc of raffinose</th>
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<tr>
<td>Fructose</td>
<td>1</td>
<td>61.393</td>
<td>62.23</td>
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<tr>
<td></td>
<td>2</td>
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<td></td>
<td>5</td>
<td>81.313</td>
<td>82.15</td>
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<td></td>
<td>6</td>
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<td>63.27</td>
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<tr>
<td>Glucose</td>
<td>1′</td>
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<td>92.90</td>
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<td></td>
<td>2′</td>
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<td></td>
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<tr>
<td></td>
<td>6′</td>
<td>65.886</td>
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<tr>
<td>Galactose</td>
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<td>98.454</td>
<td>99.29</td>
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<td></td>
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<td>6″</td>
<td>61.1</td>
<td>61.94</td>
</tr>
</tbody>
</table>

a Chemical shifts (δ) in ppm were determined relative to the internal standard sodium 4,4-dimethyl-4-silapentane-1-sulfonate (δH = δC = 0.00 ppm). b Chemical shifts (δ) in ppm were determined relative to the external standard tetramethylsilane.

**Figure 3.** Effect of pH (A) and temperature (B) on raffinose synthesis. Part (A) represents the effect of pH. All reactions were performed using 3% (w/v) washed cells for 6 h in a 1 mL reaction mixture with different 50 mmol L⁻¹ buffered solutions (sodium acetate, pH 4.0–5.5; sodium phosphate buffer, pH 6.0–7.5; Tris–HCl buffer, pH 8.0–9.0) containing 24% (w/v) sucrose and 24% (w/v) melibiose. Part (B) represents the effect of temperature. All reactions were performed using 3% (w/v) washed cells in a reaction mixture containing 24% (w/v) sucrose and 24% (w/v) melibiose for 6 h at 55 °C. Symbols: (■) fructose; (▼) glucose; (▲) sucrose; (○) melibiose; (●) raffinose. Values are the means of three replicates ± standard deviation.

**Effects of pH and temperature on raffinose synthesis**

The concentrations of raffinose produced from sucrose and melibiose by levansucrase activity in washed cells of recombinant E. coli were examined at 55 °C and pH values ranging from 4.0 to 9.0 (Fig. 3A). The maximum raffinose level, achieved at pH 6.5 (sodium phosphate buffer, 50 mmol L⁻¹), was 167 g L⁻¹, and raffinose production was significantly decreased above pH 8.0. In addition, the whole cells of recombinant E. coli displayed raffinose production on a relatively wide pH spectrum, producing more than 135 g L⁻¹ raffinose from pH 4.0 to 8.0. Raffinose was the main product in all of the reactions, whereas very small amounts of D-fructose were produced, indicating that the recombinant levansucrase had higher transfructosylation activity than sucrose hydrolysis activity in the presence of melibiose as a transfructosyl acceptor.

The effect of temperature on raffinose production was investigated with whole cells by varying the temperature from 20 to 80 °C at pH 6.5 (sodium phosphate buffer, 50 mmol L⁻¹) (Fig. 3B). The optimum temperature for raffinose production was 55 °C. The raffinose production was significantly decreased above 60 °C and completely inhibited at 80 °C due to inactivation. At 60 and 70 °C, the reactions produced moderate levels of raffinose but no D-fructose, suggesting that the recombinant levansucrase likely displayed...
transfructosylation activity in the presence of melibiose only at these temperatures.

**Effect of cell amount on raffinose synthesis**

The optimal cell amount for raffinose production was determined by varying the amount from 1% to 14% (w/v) (Fig. 4). The highest level of raffinose production was achieved by 3% (w/v) of whole cells, and more than 150 g L\(^{-1}\) raffinose was produced when the cells were added at concentrations from 2% to 5%. However, with an increase in washed whole cells (above 5% (w/v)), raffinose production was clearly inhibited and an increase in the release of D-fructose was observed, indicating that the ratio of sucrose hydrolysis to fructosyl transfer improved in the presence of excess levansucrase activity.

**Effects of substrate concentration and ratio on raffinose synthesis**

To determine the effect of substrate concentration on raffinose synthesis, two substrates were added at a 1:1 molar ratio, and raffinose production was measured by varying the total substrate concentration from 6% to 60% (w/v) (Fig. 5). When each substrate was added at 3% (w/v), the proportion of raffinose and D-fructose produced out of the total sugar content was 24% (w/v) and 7% (w/v), respectively (Fig. 5A). With the increase in total substrate concentration, the proportion of raffinose produced out of the total sugar content increased from 30% to 36% (w/v) and then was stabilized. By contrast, the D-fructose proportion decreased to below 2% when the total substrate concentration was more than 30%. High concentrations of substrates aided the fructosyl transfer reaction in producing raffinose. Additionally, the high fructosyl transfer activity was likely due to the low activity of water at high sugar concentrations, which inhibits di- or oligosaccharide hydrolysis. The effect of substrate concentration and water activity on the yield and rate of the transfer reaction was also shown for *Paenibacillus polymyxa* levansucrase, which produces lactosucrose,\(^{32}\) *Leuconostoc mesenteroides* levansucrase, which produces fructooligosaccharides,\(^{26}\) and for other glycosyltransferases that have both transfer and hydrolysis activities.\(^{33-35}\)

Herein, maximal raffinose production (221 g L\(^{-1}\)) was achieved for each substrate when the concentration was initially 30% (w/v) (Fig. 5B). Higher sugar concentrations were not tested owing to problems with solubility.

The effect of substrate concentration ratio was also investigated (Fig. 6). When sucrose concentration was fixed at 30% (w/v) while melibiose concentration was varied from 18% to 36% (w/v), the increase in melibiose concentration resulted in increased raffinose production, and the maximal raffinose production was observed at 36% (w/v) melibiose (Fig. 6A). Sucrose concentration had the same effect on raffinose production when the melibiose concentration was fixed at 30% (w/v) (Fig. 6B). Therefore, both transfer donor and acceptor concentrations had significant effects on raffinose production, and in all the tests the highest raffinose production (approximately 253 g L\(^{-1}\)) was obtained at 36/30% (w/v) or 30/36% (w/v) of sucrose/melibiose. Using a large excess of acceptor and donor glycosides at high concentrations was favorable, allowing the fructosyl transfer reaction to produce raffinose via hydrolysis by competing with water.
Biological production of raffinose from sucrose and melibiose under optimal conditions

Raffinose production using whole cells of recombinant *E. coli* harboring *C. arbusti* levansucrase activity was investigated under optimal conditions (Fig. 7). After a 5 h reaction, raffinose was rapidly produced to a level of 222 g L\(^{-1}\), and then the raffinose yield steadily declined as the reaction progressed further. During raffinose production from sucrose and melibiose, the release of D-fructose slightly but steadily increased within all the time periods tested, indicating that the recombinant levansucrase displayed increasing hydrolysis toward sucrose with the increase in reaction time. The consumption of sucrose was greater than that of melibiose, while fructose and glucose concentrations were lower than expected based upon stoichiometry of this reaction, and it was inferred that further degradation of glucose and fructose by other enzymes in *E. coli* might have occurred.

The highest raffinose yield was 222 g L\(^{-1}\), with a 50% conversion ratio from each substrate to raffinose, when 30% (w/v) sucrose and 30% (w/v) melibiose were catalyzed by 3% (w/v) whole cells at pH 6.5 (sodium phosphate buffer, 50 mmol L\(^{-1}\)) and 55 °C for 6 h. By comparison, the raffinose yield by levansucrase was remarkably higher than by *B. subtilis* levansucrase and fungal α-galactosidases. For example, the crude recombinant *B. subtilis* levansucrase produced raffinose from 0.6 mol L\(^{-1}\) sucrose and 1.2 mol L\(^{-1}\) melibiose with a conversion ratio of 45%;\(^{27}\) *A. corymbifera* α-galactosidase produced 0.17 mol L\(^{-1}\) raffinose from 1.67 mol L\(^{-1}\) D-galactose and 2.04 mol L\(^{-1}\) sucrose, with a 10% conversion ratio from D-galactose to raffinose;\(^{20}\) and *Para-pheoaephaeria* sp. α-galactosidase produced 13.3 g L\(^{-1}\) raffinose from 100 g L\(^{-1}\) D-galactose and 500 g L\(^{-1}\) sucrose.\(^{21}\)

**CONCLUSION**

The present study shows the high-yield production of raffinose from sucrose and melibiose by recombinant *E. coli* harboring *C. arbusti* levansucrase. The high substrate concentration strongly favors the production of raffinose through the fructosyl transfer reaction. This work is the first report on the optimization of reaction conditions for raffinose production by levansucrase. The conversion ratio from sucrose or melibiose to raffinose reached 50% after optimization of the reaction parameters, even when each substrate concentration reached 30% (w/v). The raffinose yield is significantly higher than that by α-galactosidase reported previously, suggesting that the reaction catalyzed by levansucrase is a more feasible approach for commercial raffinose production. Given the above high conversion rate, the higher yield may be achieved by pure levansucrase, which is isolated from the recombinant *E. coli*, combining with immobilization technics. Furthermore, considering the safety of raffinose in the food industry, the *C. arbusti* levansucrase should be expressed in the *B. subtilis* which belongs to the food-grade expression system. These studies are in progress.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES