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Biosynthesis of lactosylfructoside by an intracellular levansucrase from *Bacillus methylotrophicus* SK 21.002

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ABSTRACT

Lactosylfructoside is a functional oligosaccharide consisting of D-glucose, D-galactose, and D-fructose. In this work, lactosylfructoside was biosynthesized from sucrose as a fructosyl donor and lactose as an acceptor by an intracellular levansucrase derived from strain *Bacillus methylotrophicus* (*B. methylotrophicus*) SK21.002. The trisaccharide was purified from the product using a high performance liquid chromatography (HPLC) system and was confirmed to be lactosylfructoside by nuclear magnetic resonance (NMR) spectroscopy. The biosynthesis conditions (such as pH, temperature, enzyme dosage, substrate concentrations, and the concentration ratio of the two substrates) for lactosylfructoside production were optimized. The optimum conditions for lactosylfructoside preparation were a pH of 6.5, temperature of 37 °C, and enzyme dosage of 8 U/g substrates. The concentration of substrates (total lactose and sucrose) was 400 mg/mL, and the ratio of lactose to sucrose was 1:1. The optimum time for lactosylfructoside production was 20 h, the yield of lactosylfructoside under the optimal conditions was 143 mg/mL, and the lactosylfructoside conversion efficiency was 36%.

1. Introduction

As a type of rare trisaccharide with high quality taste similar to sucrose, lactosylfructoside (*O*-β-D-galactopyranosyl-(1,4)-*O*-α-D-glucopyranosyl-(1,2)-β-D-fructofuranoside) has been used as a prebiotic ingredient in various food products.[1] Like many other oligosaccharides, lactosylfructoside is indigestible and shows a range of physiological functions. It has the ability to maintain intestinal microflora by promoting the multiplication of *Bifidobacterium*,[2–5] inhibit fat accumulation,[6–8] and improve the absorption of intestinal calcium.[9,10] Moreover, lactosylfructoside shows a potential beneficial immunoregulatory effect.[11,12]

Barely obtained by other methods, lactosylfructoside is mainly produced through an enzymatic transglycosylation reaction with sucrose and lactose as substrates. It can be produced through a transfructosylation reaction in which the fructosyl moiety from the donor sucrose is transferred to the acceptor lactose by β-fructofuranosidase (EC 3.2.1.26) from *Arthrobacter* sp. K-1[13–15] or by levansucrase (EC 2.4.1.10) from a variety of microorganisms, including *Aerobacter leavanicum*,[16] *Paenibacillus polymyxa*,[17] *Sterigmatomyces elviae*,[18] etc.[19–22] It is known that these enzymes catalyze the transfer and hydrolysis reactions of lactosylfructoside and sucrose.[15,20] The crystal structures of β-fructofuranosidase from *Arthrobacter* sp. K-1 and levansucrase from *Gluconacetobacter diazotrophicus*, have been determined.[23,24] In addition, by transferring the galactosyl moiety from the donor lactose to the acceptor sucrose, the transgalactosylation reaction may synthesize lactosylfructoside by β-galactosidase (EC 3.2.1.23) from *Bacillus circulans*.[25,26] However, the capabilities of the three types of enzymes to produce lactosylfructoside are greatly different, even though the reactions are catalyzed by the same type of enzyme derived from different microorganisms.

A new intracellular levansucrase-producing strain *Bacillus methylotrophicus* (*B. methylotrophicus*) SK21.002 was recently screened by our laboratory[27] and is different from other extracellular levansucrases. Our research showed that the whole cells of *Bacillus methylotrophicus* SK 21.002 harbored levansucrase activity. Moreover, further research indicated that the new strain performed well in producing lactosylfructoside by levansucrase. To our knowledge, the synthesis of lactosylfructoside by levansucrase from *B. methylotrophicus* has not been previously reported. Therefore, in this study, the new strain was used to produce lactosylfructoside, and the conditions of lactosylfructoside biosynthesis from sucrose and lactose were optimized.

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2. Materials and methods

2.1. Materials

The lactosylfructoside, lactose, sucrose, glucose, and fructose standards were obtained from Sigma (St. Louis, MO, USA). Other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.2. Microorganism and medium

B. methylotrophicus SK 21.002 used in this study was previously isolated from soil of beet gardens and identified via 16S rRNA gene sequence analysis.[27] From previous studies, the optimal culture medium was determined as follows (g/L): sucrose, 80; yeast extract, 10; tryptone, 10; K_2HPO_4, 8; $MgSO_4$, 1; and NaCl, 2, while the pH was adjusted to 6.5 before autoclaving. The bacteria were grown at 30 °C for 21 h in a rotary-shaking incubator with a shaking speed of 200 rpm.

2.3. Preparation of crude levansucrase

For the preparation of crude levansucrase, the cultivated cells were collected from the fermentation broth by centrifugation at 10,000*g* for 10 min at 4 °C, washed twice with 20 mM sterile sodium phosphate buffer (pH 6.5) and then suspended in the same buffer by adding 10 mL buffer to 1 g wet cells. The cells were disrupted by ultrasonication (190 W, pulse on, 1 s; pulse off, 2 s) for 12 min. The resulting suspension was then shaken at 4 °C for 1 h to liberate the levansucrase. The unbroken cells and cellular debris were removed by centrifugation at 10,000*g* for 10 min at 4 °C. Supernatants were harvested as crude levansucrase for lactosylfructoside biosynthesis.

2.4. Activity assay of levansucrase

The levansucrase activity was determined by measuring the amount of glucose released from 20% sucrose (w/v). Assay mixtures (1 mL) containing 0.1 mL of enzyme solution, 0.4 mL of 20 mM sodium phosphate buffer (pH 6.5), and 0.5 mL of 20% sucrose (w/v) in 20 mM sodium phosphate buffer (pH 6.5) were preheated at 40 °C and incubated at 40 °C for 20 min. The reaction was stopped in boiling water for 10 min. One unit of total levansucrase activity was defined as the amount of enzyme releasing 1 μmol of glucose per minute.

2.5. Analysis of carbohydrates

The amounts of glucose, fructose, sucrose, lactose, and lactosylfructoside were analyzed by HPLC using a differential refractive index detector and a Shodex Asahipak NH_2P-50 4E column (4.6 × 250 mm), which was eluted at room temperature. As the mobile phase, 75% (v/v) acetonitrile and 25% (v/v) water were used at a flow rate of 1.0 mL/min.

2.6. Structure identification of lactosylfructoside

The above-described HPLC system was used for the purification of lactosylfructoside after biosynthesis, as followed for the reaction mixture. The biosynthesis product (0.1 mL) was injected into the HPLC system every time, and the eluate containing lactosylfructoside was collected. Then, purified lactosylfructoside for NMR analysis was acquired by lyophilization. ^{13}C and ^{1}H NMR spectra of lactosylfructoside were obtained in D_2O on a Bruker AVANCE III-400 MHz spectrometer at 30 °C. The ^{13}C NMR experiment was obtained at an operating frequency of 100.57 MHz and recorded 1402 times with a scanning width of 24038 Hz and relaxation time of 2 s. The ^{1}H experiment was obtained at an operating frequency of 400.13 MHz and was recorded 16 times with a scanning width of 8223 Hz and a relaxation time of 1 s.

2.7. Optimization of lactosylfructoside biosynthesis

To find the optimal reaction conditions for enzymatic synthesis of lactosylfructoside, the effects of pH, temperature, enzyme dosage, substrate concentrations, and concentration ratio of the two substrates were studied in batch reactions. All reactions were carried out in a 1-mL reaction mixture containing 180 mg/mL sucrose and 180 mg/mL lactose in 20 mM sodium phosphate buffer (pH 6.5) and levansucrase of 10 U/g substrates at 40 °C for 24 h and then terminated in boiling water for 10 min. In the batch-wise process, the lactosylfructoside conversion efficiency (%) was calculated by dividing the lactosylfructoside concentration by the summation of initial concentrations of sucrose and lactose.

2.7.1. Effect of pH on lactosylfructoside biosynthesis

The effect of pH on lactosylfructoside production was performed at varying pH's (5.7 to 8.0), while other conditions were the same as those of the initial reaction.

2.7.2. Effect of temperature on the lactosylfructoside biosynthesis

The optimal temperature for lactosylfructoside production was determined by incubation at 25 °C, 30 °C, 35 °C, 37 °C, 40 °C, 45 °C, and 50 °C for 24 h, respectively. Other conditions were the same as those of the initial reaction.

2.7.3. Effect of enzyme dosage on lactosylfructoside biosynthesis

To determine the optimum enzyme dosage for lactosylfructoside production, the reaction mixtures were prepared with the enzyme dosages of 2, 4, 6, 8, 10, 12, and 14 U/g substrates, while other conditions were the same as those of the initial reaction.

2.7.4. Effect of substrate concentration on lactosylfructoside biosynthesis

To determine the optimum substrate concentrations for lactosylfructoside production, the total concentration of sucrose and lactose was 100 mg/mL, 200 mg/mL, 300 mg/mL, 360 mg/mL and 400 mg/mL and both of them were used at the same concentrations. The pH, temperature, and enzyme dosage were the same as those in the optimal conditions.

2.7.5. Effect of the concentration ratio of the two substrates on lactosylfructoside biosynthesis

The effect of the concentration ratio of the two substrates on lactosylfructoside production was carried out at varying concentration ratios of the two substrates (sucrose/lactose at 1:5, 1:3, 1:2, 1:1, 2:1, 3:1, and 5:1), while the total concentration of the two sugars was fixed at 400 mg/mL. The pH, temperature, and levansucrase dosage were maintained at the optimal conditions.

2.8. Lactosylfructoside biosynthesis

Enzymatic synthesis of lactosylfructoside from sucrose and lactose by levansucrase from *B. methylotrophicus* was investigated under the optimum conditions. The reactions were performed at different time intervals.

The concentration of lactosylfructoside was expressed as the mean ± standard deviation ($n = 3$).