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Simple, fast, and efficient process for producing and purifying trehalulose

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ABSTRACT

A new property of recombinant trehalose synthase (GTase) from *Thermus thermophilus* HB-8 (ATCC 27634) was found and described in this study. GTase can act on sucrose and catalyze trehalulose formation without isomaltose, isomaltulose, or isomelezitose, releasing small amounts of glucose and fructose as byproducts. Maximum trehalulose yield (approximately 81%) was obtained at an optimum temperature of 65 °C and was independent of substrate concentration. A simple, fast, and efficient method of producing and purifying trehalulose is then described. In the first step, GTase catalyzed trehalulose formation using a 20% sucrose substrate. Miscellaneous sugars were then rapidly removed, while trehalulose was completely preserved by *Saccharomyces cerevisiae* cells. Finally, the cells were separated by centrifugation, and salt ions were removed by an ion-exchange resin, subsequently obtaining a high-purity trehalulose solution. A trehalulose recovery rate of over 95% was achieved using this process. This method has a simple process, fast separation efficiency, and low investment in production equipment, so greatly to improve production efficiency and reduce production costs.

1. Introduction

Trehalulose (1-O-α-D-glucosylpyranosyl-β-D-fructose) is a reducing disaccharide that is naturally present in small quantities in honey (Low & Sporns, 1988; Nakajima, Sugitani, Tanaka, & Fujii, 1990). It is a structural isomer of sucrose (α-D-glucosylpyranosyl-1,2-β-D-fructofuranoside) and has been proven to have two optical isomers, namely, 1-O-α-D-glucosylpyranosyl-β-D-fructofuranose and 1-O-α-D-glucopyranosyl-β-D-fructopyranose (Cookson, Cheetham, & Rathbone, 1987). The sweetness of this sugar is approximately 60% that of sucrose; however, it is non-cariogenic (Ooshima et al., 1991) and shows a slower rate of monosaccharide release into the blood. Trehalulose has high solubility (Ooshima et al., 1991) and exhibits physiological functions and chemical properties similar to those of isomaltulose and trehalose. It can prevent dental cavities, mitigate diabetes mellitus, and help maintain body weight (Minami, Fujiwara, Ooshima, Nakajima, & Hamada, 1990; Ooshima et al., 1991). The growing list of disorders related to cardiovascular pathologies, several forms of cancer, diabetes, obesity, osteoporosis, and infectious dental diseases are associated with excessive sugar intake. Therefore, the development of a healthy sugar substitute is urgently needed (Ravaud et al., 2007). Trehalulose is a promising substance that has a wide range of potential applications in the food, cosmetics, pharmaceutical, and other industries.

The chemical synthesis of trehalulose is highly difficult, and its industrial production proceeds exclusively from sucrose using immobilized microorganisms. Several organisms or enzymes (including trehalulose synthase, isomaltulose synthase, and sucrose isomerase) can convert sucrose into trehalulose, or a mixture of isomaltulose and trehalulose to produce glucose and fructose in residual amounts through sucrose hydrolysis. Depending on the enzyme, the composition of enzyme products varies from mainly isomaltulose (66–91%) (Cheetham, 1984; Véronèsea & Perlot, 1999; Wu & Birch, 2005; Zhang, Li, & Zhang, 2002) to predominantly trehalulose (~90%) (Nagai, Sugitani, & Tsuyuki, 1994). In particular, sucrose isomerase from whiteflies converts sucrose into mainly trehalulose, without isomaltulose (Salvucci, 2003). However, the paper did not report trehalulose yield. Hamerli and Birch (2011) developed sugarcane plants to produce trehalulose through the expression of a vacuole-targeted trehalulose synthase modified from a gene of "*Pseudomonas mesoacidophila* MX-45". The trehalulose concentration in the juice increased with internode maturity, reaching approximately 600 mM and a near-complete sucrose conversion in the most mature internodes. The plants remained vigorous, and trehalulose production in selected lines was retained over multiple vegetative generations under glasshouse and field conditions.

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Complex products are generated during trehalulose production, including a considerable number of impurities. This condition, coupled with high solubility, renders the separation, purification, and crystallization of trehalulose difficult. Moreover, currently available reports on separation and purification methods are inadequate. Véronèse, Bouchu, and Perlot (1999) and Kishihara et al. (1989) obtained high-purity trehalulose via preparative chromatography and through the use of a simulated moving-bed adsorber (Kishihara et al., 1989; Véronèse et al., 1999). Cookson et al. (1987) purified trehalulose from a complex mixture using four reversed-phase preparative-scale HPLC columns, with distilled water as the eluent, achieving a final purity of 98%. The mixture, which was produced by immobilized microbial cells, consisted of a trisaccharide, sucrose, isomaltulose, glucose, and fructose. However, the high cost and low yield of the aforementioned separation and purification methods make them unsuitable for commercial production.

Zdziebło and Synowiecki (2006) and Wang et al. (2012), reported the properties of a trehalose synthase (GTase or TST) from *Thermus thermophilus* HB-8 (ATCC 27634). The enzyme catalyzes the conversion of maltose into trehalose at an optimum temperature of 65 °C. In the current paper, a new property of GTase is described. The sucrose products under the catalysis of GTase were confirmed via high-performance liquid chromatography (HPLC). Given the substrate availability and higher product yield, this enzyme is suitable for the industrial production of trehalulose from sucrose. Thus, a simple, fast, and efficient process of producing and purifying trehalulose is proposed.

2. Materials and methods

2.1. Bacterial strains, plasmids and reagents

Saccharomyces cerevisiae CICC1001 obtained from CICC (China) and *T. thermophilus* HB8 (ATCC27634) were used in the current study. *Escherichia coli* JM109 (Promega, USA) was used for routine cloning, whereas *E. coli* BL21 was used for gene expression. Plasmid pSE380 (Invitrogen, USA) was used as a cloning and expression vector. *E. coli* and recombinant *E. coli* strains were routinely grown at 37 °C and 200 rpm either in a Luria–Bertani medium alone or with 100 μg/ml ampicillin. The restriction enzymes, ligase, and LA Taq DNA polymerase were all obtained from TaKaRa (Shiga, Japan). The 732 cation exchange resin and 717 anion exchange resin (Product Nos. 10024260 and 10024160) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Trehalulose was obtained from Wako (Japan). All other saccharides were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Construction of recombinant expression vectors

All experimental methods were in accordance with previously described techniques (Wang et al., 2012). The expression vectors were linked with 6× His-tag at the C terminus for purification in a nickel–nitriloacetic acid (Ni–NTA) column (Qiagen, Hilden, Germany). The recombinant plasmid (pSE380-GTase) was transformed into *E. coli* BL21 for GTase expression.

2.3. GTase expression and purification

After the cell density (A_{600}) of the incubated recombinant cells reached approximately 0.6, isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce gene expression. The cells were harvested via centrifugation at 8000*g* for 10 min, washed twice with 50 mM potassium phosphate buffer (pH 7.0), resuspended in the same buffer, and then disrupted by sonication on ice for 10 min after 10 h induction at 37 °C. Cell debris were removed through centrifugation at 12,000*g* for 20 min. The recombinant protein was purified using Ni–NTA (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Enzyme protein content was determined using the Bradford method (Bradford, 1976), with BSA as standard.

2.4. Preparation of S. cerevisiae cells

S. cerevisiae was grown in a yeast extract–peptone–glucose (YPD) medium (pH 7.0) containing 2% peptone and 1% yeast extract at 30 °C and 180 rpm. After the A_{600} of the incubated *S. cerevisiae* cells reached approximately 3.0, the cells were harvested by centrifugation at 8000*g* for 10 min and washed twice with distilled water.

2.5. Enzyme assay

The GTase reactions in a final volume of 500 μl involved incubation at 65 °C for 2 h in a reaction mixture containing 0.5 mg of purified enzyme, 2% sucrose, and 50 mM phosphate buffer (pH 7.0). A volume of distilled water equal to that of the purified enzyme was added to the reaction mixture as a control. The identity of the sucrose products obtained under GTase catalysis was confirmed via HPLC, which was performed at 30 °C and a pressure of 76 bar using an Agilent 1100 series column (Alltima Amino 100A 5u, 250 mm × 4.6 mm) and an Alltech 2000ES evaporative light scattering detector. Acetonitrile/water (76:24) was used as solvent at a flow rate of 1 ml min^{-1}. Enzyme activity was assayed by measuring the amount of consumed substrate via HPLC, using the control as the original substrate content. One unit of enzyme activity was defined as the amount of enzyme that consumed 1 μmol of substrate per minute.

Kinetic analysis was performed at an optimum temperature of 65 °C and a pH of 7.0. The experiment was conducted for 1 h in a 50 mM phosphate buffer containing the sucrose substrate at various concentrations. The K_m and V_{max} values were obtained using a Lineweaver–Burk plot.

2.6. Simple process sequence of trehalulose production and purification

A simple process sequence is outlined in Fig. 1. First step: Trehalulose production involved incubation of a reaction mixture containing 90 mg of GTase protein, 20 g of sucrose, and 50 mM of phosphate buffer (pH 7.0) at 65 °C. The generated trehalulose was monitored by HPLC. The reaction solution was then boiled for 10 min, and the enzyme was removed by centrifugation at 12,000*g* for 10 min to obtain a coarse trehalulose solution. Second step: Harvested *S. cerevisiae* cells were resuspended in the coarse trehalulose solution (w/v = 1 g/10 ml), and then transferred to a 500 ml culture bottle for culturing at 30 °C and 180 rpm. Changes in fructose, glucose, and sucrose amounts were monitored in real-time via HPLC. *S. cerevisiae* cells were immediately removed by centrifugation at 12,000*g* for 10 min when fructose, glucose, and sucrose were completely digested. The obtained solution consisted of a pure trehalulose solution; however, a small amount of salt ions and impurities were also present. Third step: Cation and anion exchange resin were used to separate and purify trehalulose from salt ions and impurities. The pretreatment using the ion-exchange resin was in accordance with the instructions of the manufacturer. The processed filler was filled into a 2 cm × 1.5 m chromatography column. Distilled water was used as the mobile phase at a flow rate of 1 ml/min for approximately 2 h for chromatography column equilibration. Under the same conditions, the