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Overexpression of secreted sucrose isomerase in *Yarrowia lipolytica* and its application in isomaltulose production after immobilization

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ABSTRACT

Isomaltulose production by bacterial fermentation was limited, due to generation of undesirable products and reduced yields. Isomaltulose production using sucrose isomerase (SIase) catalyzed methods was expected to be more applicable, but was hampered by low SIase activity and lack of a secreted SIase producer. Here, we aimed to obtain high levels of secreted SIase by overexpressing the SIase gene from *Pantoea dispersa* UQ68J in *Yarrowia lipolytica*, a successful host for efficient secretory expression, with a newly characterized strong constitutive promoter. After optimization of the culture medium, the engineered strain JD secreted SIase with an activity of 49.3 U/mL. The recombinant SIase was effectively immobilized onto polyvinyl alcohol-alginate, and the enzymatic activity recovery rate was up to 82.4%. The stability of the SIase was significantly improved by immobilization. Batch production of isomaltulose catalyzed by the immobilized SIase was performed under optimal conditions, generating 620.7 g/L isomaltulose with a yield of 0.96 g/g. The conversion rate of sucrose after 13 batches remained above 90%. These results demonstrated that the proposed SIase expression and immobilization method was promising in the industrial production of isomaltulose.

1. Introduction

Sucrose (α D glucopyranosyl 1,2 D fructofuranose) is a commonly used sweetener. Upon ingestion, sucrose is degraded into glucose and fructose, thus elevating blood glucose levels [1]. Excessive sucrose intake can easily cause obesity and is associated with health problems, such as heart disease and type 2 diabetes [1]. Increases in the standard of living and health consciousness have led to a focus on healthier functional sweeteners that can replace sucrose [2,3]. Isomaltulose is an ideal functional sweetener that has been approved as a safe substitute for sucrose by the United States Food and Drug Administration [4]. This compound also possesses health advantages, including prolonged energy release, slower digestion, higher stability, lower carcinogenicity, and reduced insulin levels [5].

In industry, isomaltulose is mainly produced from sucrose by bacterial fermentation. Those isomaltulose producing strains can synthesize sucrose isomerase (SIase), which is responsible for isomerizing sucrose into isomaltulose and other by-products, such as trehalulose, fructose, and glucose [6–11]. But the concentration of sucrose substrate was limited and the isomaltulose yield was reduced during the fermentation, as sucrose serves as both substrate of the SIase and carbon source for cell growth [11–13]. Even using free cells or immobilized cells to produce isomaltulose can't avoid the extra sucrose consumption via cell utilization [6–12]. Additionally, those isomaltulose producing strains lack a food-grade genetic background. The existence of various uncertified extracellular products complicated isomaltulose recovery process [11].

Implementing the isomerization reaction using pure SIase was expected to be more applicable for isomaltulose production, for the increased substrate concentration and separated products from strains [11]. Immobilization of SIase has been tried to achieve repeated use of enzymes and improved stability, reducing enzyme demand [14,15]. Generally, wild strains secrete SIases with low activity, not meeting the demand of isomaltulose production [7,9–11]. High SIase activity was obtained by expressing SIase genes in *Escherichia coli*; but the intracellular localization of SIases and endotoxin generation necessitate expensive and difficult downstream isolation processes [11,16,17]. Secretory expression of the SIase gene has been tried in *Lactococcus lactis*. However, the activity was below 3.0 U/mL, far from meeting industry requirements [13]. Accordingly, lack of a suitable secreted SIase

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producer should be a significant obstacle to isomaltulose production using enzymatic methods. Thus, a more suitable host should be identified for secretory expression of SIase.

The yeast *Yarrowia lipolytica* is classified as a "generally recognized as safe" microbe and has been shown to be a successful host for efficient secretory expression of many bacterial and fungal genes [18]. TEF promoter with intron (TEFin) is a newly characterized strong constitutive promoter in *Y. lipolytica*, inducing a 5-fold increase in expression over the traditional promoter [19]. In this study, we aimed to obtain high levels of secreted SIase and achieve isomaltulose production by enzymatic methods. To this end, the SIase gene from *P. dispersa* UQ68J with highest product specificity, was ligated into the expression vector pM06 carrying TEFin promoter, and transformed into *Y. lipolytica* host. Then the secreted SIase was immobilized onto polyvinyl alcohol (PVA), a support of low cost and nontoxicity [20], to implement isomaltulose production using enzymatic methods.

2. Materials and methods

2.1. Strains, plasmids, and media

The uracil mutant *Y. lipolytica* XY strain was kindly provided by Dr. Xiao-Yan Liu (Huaiyin Normal University). The expression vector pM06 was kindly provided by Ji-Ming Wang (Qingdao Institute of Bioenergy and Bioprocess Technology); the plasmid and accessory DNA elements were shown in Fig. S1. *E. coli* DH5α (TaKaRa Biotechnology, China) was used for plasmid amplification. *E. coli* was cultured at 37 °C in LB medium supplemented with 30.0 μg/mL kanamycin or 100.0 μg/mL ampicillin. Yeast strains were cultivated in YPD medium (20.0 g/L glucose, 20.0 g/L peptone, 10.0 g/L yeast extract) [18]. *Y. lipolytica* JD transformants were screened on YNB plates (1.7 g/L yeast nitrogen base without amino acids, 10.0 g/L glucose, 5.0 g/L $(NH4)_2SO_4$, 25.0 g/L agar) [18].

2.2. DNA manipulation

Restriction endonuclease digestion and DNA ligation were performed according to Thermo Scientific protocols. DNA polymerase was used as recommended by the manufacturer (New England BioLabs, USA). Digested DNA fragments were recovered and purified using a TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0. *E. coli* was transformed was performed as previously described [21]. *E. coli* transformants were screened on LB agar containing 30.0 μg/mL kanamycin or 100.0 μg/mL ampicillin. Genomic DNA from corresponding *Y. lipolytica* transformants was extracted using a TIANamp Yeast DNA Kit (TIANGEN BIOTECH, China).

2.3. SIase expression in Y. lipolytica

The SIase gene from *P. dispersa* UQ68J (accession no.: AY223549) was used for recombinant SIase expression. After codon redesign and optimization, the coding sequence of the SIase gene with or without a 6× His tag was synthesized (Synbio Technologies, China), named *PDSIH* and *PDSI*, respectively. The synthesized *PDSIH* and *PDSI* DNA fragment with *Sfi*I and *Bam*HI restriction sites was ligated into pMD19-T, forming pMD19-T-*PDSIH*, pMD19-T-*PDSI* (Synbio Technologies). The plasmids pMD19-T-*PDSIH*, pMD19-T-*PDSI*, and pM06 were digested by *Sfi*I and *Bam*HI. After purification, the fragment containing *PDSIH* or *PDSI*, and linearized pM06 were ligated to construct the final recombinant expression plasmid pM06-*PDSIH* and pM06-*PDSI*.The two expression plasmids were linearized following *Not*I digestion. The purified fragments carrying SIase gene were separately transformed into *Y. lipolytica* URA^- cells using the LiAc method [22]. The linearized fragment of pM06 not carrying any genes was transformed into the host as a control. Positive transformants were grown on YNB agar at 30 °C. To confirm integration of the SIase coding gene, genomic DNA from the transformants and control strain was extracted and verified by polymerase chain reaction (PCR) using the following primers: SIR (5′-AACCATTGTCGGTGTTAGGAGAC-3′) and URAF (5′-AAGAAACCGTGCTTAAGAGC-3′).

2.4. Purification of the recombinant SIases

Based on PCR detection, a transformant named strain JD carrying *PDSIH* gene was picked, the gene coding SIase with a 6× His tag. And a transformant named strain JW carrying *PDSI* gene was also picked, the gene coding SIase without a 6× His tag. Strain JD and strain JW were separately cultivated in YPD liquid seed medium for 20 h and then switched to 100 mL YPD liquid fermentation medium for 60 h. Cultures were centrifuged at 5000 ×*g*, and supernatants were collected for analysis of SIase activity. The fermentation broth of strain JD and strain JW were concentrated 10 times using a 10-kDa ultrafiltration membrane. These concentrated supernatants containing the SIase enzyme were dialyzed overnight.

The concentrated supernatant of strain JW containing the SIase enzyme PDSI was adjusted to pH 6.0 in phosphate buffer (50 mM), and then applied to a DEAE Sepharose™ Fast Flow column (GE Healthcare, USA). The SIase enzyme PDSIH was attached to the gel and then eluted with a linear gradient NaCl solution (0–1 M) at a flow rate of 1 mL/min. The concentrated supernatant of strain JD containing the SIase enzyme with 6× His tag was adjusted to pH 5.0 using in phosphate buffer (50 mM), then applied to a Ni-chelating affinity gel column (GE Healthcare, USA). The SIase enzyme PDSIH was attached to the gel and were eluted stepwise, each time with 20 mL of elution buffer (20 mM Tris, pH 8.0, 500 mM NaCl, imidazole), with imidazole concentrations ranging from 10 to 500 mM. The SIase enzyme solutions were dialyzed and stored at −80 °C. The protein concentration was determined by the Bradford method with bovine serum albumin (BSA) as standard. The purity and molecular mass of the enzymes were verified by SDS-PAGE.

2.5. Enzymatic activity assay and sugar concentration determination

SIase activity was detected as described previously [16]. Briefly, 100 g/L sucrose was prepared in phosphate buffer (50 mM, pH 6.0). Then, 100 μL SIase solution or immobilized SIase was added, and the total volume was adjusted to 1.0 mL after incubation at 30 °C for 10 min. The final reaction solutions were filtered through a 0.22-μm membrane and diluted for high performance liquid chromatography (HPLC) analysis. The samples were injected onto an amino column (Thermo Scientific, USA) fitted on an Agilent 1200 system (Agilent Technologies, USA). Sugar concentrations were determined using a refractive index detector. The mobile phase was acetonitrile: water (88:12) with the flow rate of 1.5 mL/min at 30 °C. The amounts of isomaltulose, trehalulose, glucose, fructose, and sucrose were calculated according to peak areas and retention time. One unit of SIase activity was defined as the amount of enzyme releasing 1 μmol isomaltulose or trehalulose in 1 min under the catalyzing conditions mentioned above.

2.6. Optimization of culture medium for SIase production in flasks

Initial SIase producing YPD medium contained 20.0 g/L glucose, 20.0 g/L peptone, and 10.0 g/L yeast extract (pH 6.0). All batch fermentations in flasks were conducted for 72 h at 30 °C and 180 rpm. Different nitrogen sources and nitrogen source concentrations were evaluated. Remaining glucose was detected by HPLC as described above; cell dry weight was evaluated as described previously [23].