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High-level secretion and characterization of cyclodextrin glycosyltransferase in recombinant *Komagataella phaffii*

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ABSTRACT

Cyclodextrin glycosyltransferase (CGTase) catalyzes the conversion of starch into cyclodextrin (CD), which is widely applied in food, pharmaceutical, cosmetic, and agricultural industries. For efficient production of CD, high yield of CGTase with good characteristics is necessary. In this study, the *cgt* gene from *Bacillus pseudalcaliphilus* was expressed in *Komagataella phaffii* after codon optimization and expression vector selection. The β-CGTase activity in the transformant reached 3885.1 U mL^{-1}, which is the highest value reported so far, at 28 °C, 6% inoculum ratio, and 1.5% methanol addition following 24 h of incubation. The recombinant CGTase showed high specific activity at 80 °C without any γ-CGTase activity, and had good stability in a wide pH and temperature range. These results demonstrate that the recombinant CGTase could have potential industrial applications.

1. Introduction

Cyclodextrins (CD) are a family of cyclic oligosaccharides with 6, 7, and 8 glucopyranose units, named as α-CD, β-CD, and γ-CD, respectively (Crini, 2014). The most important characteristic of CDs is their asymmetric hydrophobic cavity that can be employed to recognize molecules through noncovalent bonds such as hydrogen bonds, ionic bonds, van der Waals forces, and hydrophobic interactions. CDs and their derivatives have various applications (Sharma and Baldi, 2016) in biotechnology (He et al., 2015), cosmetics and personal care (Mathapa and Paunov, 2013), food (Fenyvesi et al., 2016), pharmaceuticals (Swaminathan et al., 2016), and chemical industries (Sharma and Baldi, 2016). Currently, enzymatic synthesis is the major approach employed for the production of CDs (Biwer et al., 2002; Jin, 2013), which requires large amount of cyclodextrin glycosyltransferase (CGTase) with high specific activity (Ng et al., 2013). As a result, CGTase produced from microbial sources with high specific activity is always in demand for effective production of CDs (Kitayska et al., 2011). It has been reported that about 50% of commercial CGTases are produced by genetic engineering (Qi and Zimmermann, 2004). These CGTases have diverse properties and are expressed in microbial hosts with or without mutation, such as in *Escherichia coli* (Li et al., 2010), *Bacillus subtilis* (Jeang et al., 2005), and *Saccharomyces cerevisiae* (Wang et al., 2006), for overproduction (Han et al., 2014). Although remarkable progress in CGTase overproduction has been achieved during the past decades, further research on heterologous expression is needed to improve CGTase production. *Komatagaella phaffii*, formerly *Pichia pastoris*, (Kurtzman, 2005, 2009) has certain characteristics that make it an attractive host for high-level heterologous protein production, such as well-established vectors and protocols, a strict regulation system of alcohol oxidase 1 (AOX1) promoter, high transcriptional activity (Prielhofer et al., 2015), secretory expression patterns, and easy cultivation with high cell density. To date, many heterologous genes have been expressed in *P. pastoris* (Kim et al., 2015), and several proteins produced using this microorganism has been approved by the US Food and Drug Administration (FDA) as Generally Recognized as Safe (GRAS) products to be released into the market (Gasser et al., 2013; Vogl et al., 2013). In addition, humanized glycosylation of heterologous proteins from *P. pastoris* (Bretthauer, 2003) and genomic analysis (De Schutter et al., 2009) (Love et al., 2016) are beneficial to high-level expression and wide applications of proteins derived from *P. pastoris*.

In the present study, the *cgt* gene from *Bacillus pseudalcaliphilus* 8SB was optimized and expressed in *K. phaffii* (formerly known as *P. pastoris* GS115). The β-CGTase activity was used as an indicator for recombinant *K. phaffii* screening because the β-CGTase activity of *B. pseudalcaliphilus* 8SB CGTase is higher than the γ-CGTase activity, which has significant potential for β-CD production. The results revealed that CGTase activity was significantly increased after codon optimization, expression vector selection, and culture condition optimization. In addition, the specificity and activity of CGTase was enhanced by temperature and pH optimization, indicating the potential of this enzyme for high-purity CD production.

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2. Materials and methods

2.1. Chemicals and biochemicals

The *cgt* gene from *B. pseudalcaliphilus* 8SB (Petrova et al., 2012) was optimized according to the codon adaptation index (CAI) in Codon Usage Database (http://www.kazusa.or.jp/codon/) without altering its amino acids sequence (Supplemental Fig. 1). It has an open reading frame (ORF) of 2112 bp encoding a polypeptide of 704 amino acids, including a 29-amino-acid signal sequence and a 675-amino-acid mature enzyme (which was noted to be 71 kDa in SDS-PAGE). All the primers and *cgt* used in this study were synthesized by Shanghai Generay Biotech Co., Ltd. (Shanghai, China). *K. phaffii* and zeocin were purchased from Life Technologies (Carlsbad, CA, USA). All the restriction enzymes, DNA markers, and protein markers used in this study were purchased from Takara Biotechnology Co., Ltd (Dalian, China). All the chemicals were purchased from Sino Chemical Company (Shanghai, China).

2.2. Media

YPD medium contained the following (per L): yeast extract, 10 g; peptone, 20 g; glucose, 20 g; and agar powder (if necessary), 15 g. BMGY medium comprised the following (per L): yeast extract, 10 g; peptone, 20 g; PBS buffer (pH 7.0), 100 mmol; glycerol, 20 g; YNB, 13.4 g; and biotin, 4×10^{-4} g. BMMY medium was composed of the following (per L): yeast extract, 10 g; peptone, 20 g; PBS buffer (pH 7.0), 100 mmol; methanol, 5 mL; and biotin, 4×10^{-4} g. MD medium contained the following (per L): YNB, 13.4 g; biotin, 4×10^{-4} g; and glucose, 20 g. YPDS medium comprised the following (per L): yeast extract, 10 g; peptone, 20 g; glucose, 20 g; sorbitol, 1 mol; and agar powder (if necessary), 15 g (Cregg, 2007).

Starch solution was prepared by dissolving 4 g of soluble starch in 66.67 mmol L^{-1} phosphate buffer to a final volume of 100 mL. Phenolphthalein solution was prepared by dissolving 0.02 g of phenolphthalein in 100 mL of 5 mmol L^{-1} Na_2CO_3 solution. Bromocresol green solution contained 0.35 g of bromocresol green dissolved in 20% ethanol to a final volume of 100 mL.

2.3. Expression of cgt in K. phaffii

The *cgt* gene without signal sequence was ligated to three vectors (pPIC9K, pPICZαA, and pPICZA) after restriction endonucleases digestion, whereas the *cgt* gene with signal sequence was ligated to pPICZA after restriction endonucleases digestion, according to the molecular cloning protocol (Green and Sambrook, 2012) and *K. phaffii* protocol (Cregg, 2007). After linearization, the recombinant vector was integrated into the *K. phaffii* genome using homologous recombination via gene insertion or gene replacement of *K. phaffii* genes. Restriction enzyme *Sac*I did not digest the *aox*1 sequence of the recombinant vector, which resulted in gene insertion during transformation (methanol utilizing plus type, Mut^+), whereas the restriction enzyme *Bgl*II digested the *aox*1 sequence of the recombinant vector, leading to aox1 gene replacement during transformation (methanol utilizing slow type, Mut^S). The presence of *cgt* ORF was verified by sequencing. The *cgt* gene and vectors pPIC9K, pPICZαA, and pPICZA were digested using restriction endonucleases (*Eco*RI and *Not*I) simultaneously, and mixed to generate recombinant vectors pPIC9K-*cgt,* pPICZαA-*cgt,* and pPICZA-*cgt,* respectively. Similarly, the *cgt* gene with signal gene (*sig*) was also ligated to pPICZA to generate the recombinant vector pPICZA-*sig*-*cgt*. All the recombinant vectors were transformed into *E. coli* DH5α cells for amplification. Subsequently, the vectors (4–5 μg) pPIC9K-*cgt,* pPICZαA-*cgt,* pPICZA-*cgt,* and pPICZA-*sig*-*cgt* were digested by restriction endonuclease (*Sac*I) for linearization, and employed to transform electrocompetent *K. phaffii* cells (80 μL) using Gene Pulser (Bio-Rad, Hercules, CA, USA) at 1.1 kV, 200 Ω, and 25 mF (Higgins and Cregg, 1998). Similarly, pPIC9K-*cgt* was also linearized using *Bgl*II for transformation. The Mut^+ and Mut^S transformants were screened on histidine-deficient MD plates (and with PCR when pPIC9K-*cgt* was used). The recombinant *K. phaffii* transformants with high copy number of *cgt* gene were obtained by screening on YPD plates with different concentrations of geneticin (0.5, 1.0, 2.0, 3.0, and 4.0 mg mL^{-1}). The *cgt* gene copy number of *K. phaffii* transformants with the highest β-CGTase activity was identified using real-time PCR.

2.4. Selection of K. phaffii **transformants with high CGTase activity**

A total of 20 clones of *K. phaffii* transformants with pPIC9K-*cgt*-*Sac*I, pPIC9K-*cgt*-*Bgl*II, pPICZαA-*cgt*, pPICZA-*cgt*, and pPICZA-*sig*-*cgt,* respectively, were selected based on geneticin resistance, inoculated into 4 mL of BMGY medium, and incubated at 30 °C and 180 rpm for 24 h. Subsequently, the cells were collected by centrifugation and inoculated into 3 mL of BMMY medium in a 15-mL glass tube, to which 0.5% methanol was added every day to induce CGTase expression. The CGTase activities of the pPIC9K-*cgt*-*Sac*I, pPIC9K-*cgt*-*Bgl*II, pPICZαA-*cgt*, and pPICZA-*sig*-*cgt* transformants in the fermentation broth were measured after 72 h of induction, while those of the pPICZA-*cgt* transformants were measured after 72 h of induction and cell lysis using glass beads. Subsequently, to confirm CGTase activity and identify the phenotype, 13 clones of *K. phaffii* transformants were selected based on their β-CGTase activity and analyzed.

2.5. Determination of cgt copy number by quantitative real-time PCR

Quantitative real-time PCR (7900HT Fast Real-Time PCR System, Applied Biosystems, Foster City, CA, USA) was conducted using glyceraldehyde-3-phosphate dehydrogenase (*gap*) gene (GenBank: U62648.1) in *K. phaffii* genome as the house-keeping gene. The PCR was performed with SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio. Inc. Dalian, China) according to the manufacturer's protocol. The reaction mixture (25 μL) contained 2 μL of genomic DNA (4 ng), 12.5 μL of SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio. Inc.), 1.0 μL of each primer (10 μmol L^{-1}), and 8.5 μL of dH_2O. The DNA concentrations were measured using a spectrophotometer at 260 nm (2000c, Nano Drop, Wilmington, DE USA). The amplification reaction profile included a hotstart at 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s, 55 °C for 20 s for *gap* or 50 °C for 20 s for *cgt*, and 72 °C for 20 s. A 10-fold dilution series of the PCR product ranging from 10^7 to 10^3 copies per reaction was used. Normalization of the *cgt* gene amplification efficiency was achieved by using *gap* as the reference gene for quantification. The *cgt* gene copy number was calculated by dividing the *cgt* gene amplification efficiency by *gap* gene amplification efficiency because the copy number of *gap* in the genome was 1.

2.6. Optimization of K. phaffii cultivation conditions

K. phaffii cells were cultivated in 50 mL of YPD medium in 250-mL flasks at 30 °C and 200 rpm for 18–24 h, up to an OD_{600} of about 2.0–8.0. Subsequently, the cells were transferred into 25 mL of BMGY medium in 250-mL flasks at an inoculum ratio of 4% and incubated at 200 rpm for 24 h. Then, the culture medium was replaced with 50 mL of BMMY medium in 250-mL flasks after centrifugation according to the *K. phaffii* protocol, and 0.5% methanol was added every 24 h to induce *cgt* expression. The default culture conditions were 30 °C, 0.5% methanol, and a 4% inoculum ratio. The temperatures examined were 26 °C, 28 °C, and 30 °C; inoculum ratios investigated were 2%, 4%, 6%, 8%, and 10%; and methanol concentrations analyzed were 0.25%, 0.5%, 1.0%, 1.5%, and 2%.

2.7. Lysis of K. phaffii cells

A total of 0.5 mL of cell broth was transferred into an Eppendorf

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