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Production of γ-cyclodextrin by *Bacillus cereus* cyclodextrin glycosyltransferase using extractive bioconversion in polymer-salt aqueous two-phase system

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Aqueous two-phase system (ATPS) extractive bioconversion provides a technique which integrates bioconversion and purification into a single step process. Extractive bioconversion of gamma-cyclodextrin (γ-CD) from soluble starch with cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) enzyme derived from *Bacillus cereus* was evaluated using polyethylene glycol (PEG)/potassium phosphate based on ATPS. The optimum condition was attained in the ATPS constituted of 30.0% (w/w) PEG 3000 g/mol and 7.0% (w/w) potassium phosphate. A γ-CD concentration of 1.60 mg/mL with a 19% concentration ratio was recovered after 1 h bioconversion process. The γ-CD was mainly partitioned to the top phase (Y_T = 81.88%), with CGTase partitioning in the salt-rich bottom phase (K_{CGTase} = 0.51). Repetitive batch processes of extractive bioconversion were successfully recycled three times, indicating that this is an environmental friendly and a cost saving technique for γ-CD production and purification.

[**Keywords:** Aqueous two-phase system; *Bacillus cereus*; Cyclodextrin; Cyclodextrin glycosyltransferease; Extractive bioconversion]

An aqueous two-phase system (ATPS) is formed by mixing two incompatible aqueous solutions at a concentration exceeding a specific threshold (1). The ATPS provides a simple, low cost, and low pollution technique for purification, and it has proven to provide a biocompatible environment for the separation since both phases consist of a predominantly high water content (2,3). In this study, the production of γ-CD was performed using the enzymatic bioconversion method. Results of research on bioconversion have been used to facilitate the production of other bioproducts such as β-D-glucose-1-phosphate (4), naringin (5), bioethanol (6), L-DOPA (7), and curcuminoids (8). The application of ATPS extractive bioconversion provides a technique which integrates bioconversion and purification into a single step process (9). ATPS extractive bioconversion was designed to partition the biocatalyst and the desired product into the bottom and top phase respectively. In contrast with conventional methods (e.g., enzymatic bioconversion), ATPS extractive bioconversion integrated production and purification process into single step, the biocatalyst retained in one phase can be reused, prompting the possibility of a continuous extractive bioconversion process (Fig. 1) (10).

The Cyclodextrins (CDs) are cyclic oligosaccharides produced by cyclodextrin glycosyltransferase (CGTase) enzyme via the transglycoslation process (11). CDs have a structural feature which enables them to form inclusion complexes with a lot of guest compounds, promoting wide spread applications of CDs in different industries such as food, pharmaceuticals, cosmetics, agricultural and chemical products (12).

There are three major types of CDs, namely, α, β and γ which are formed by 6, 7 and 8 glucopyranose units, respectively (13). Among these three types of CDs, the γ-CD has the premier solubility and the largest interior cavity. In addition, γ-CD is more expensive than α and β-CD due to its lower production compare with α and β-CD (14,15). The extractive bioconversion of cyclodextrins (α-CD, β-CD and γ-CD) by *Bacillus cereus* cyclodextrin glycosyltransferase in poly (ethylene glycol) [PEG] 20,000 and dextran T500 aqueous two-phase system has been explored (16). Unfortunately, there is no available study on ATPS extractive bioconversion which focuses on optimized production of γ-CD as target product. Motivated by this, an ATPS extractive bioconversion is studied in this work to

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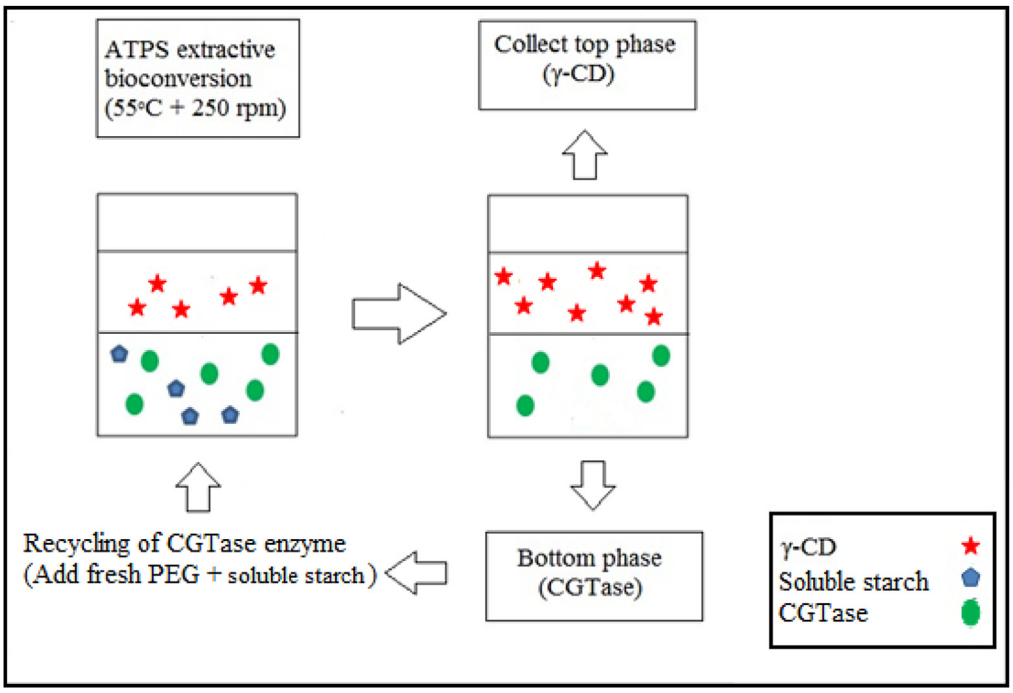

FIG. 1. Schematic diagram of ATPS extractive bioconversion.

investigate ways of improving the productivity and separation of γ-CD from *B. cereus* CGTase (15,16). The way of optimizing the γ-CD recovery were studied by investigating the effects of ATPS variables such as the pH (pH 6.0, 6.5, 7.0, 7.5, 8.0), tie-line lengths (TLLs) (27.2–40.7 %, w/w) and volume ratio (V_R) (V_R = 0.3, 1.0, 2.0, 2.3, 4.0 and 5.0). PEG with molecular weight 3000 g/mol was selected in this study.

MATERIALS AND METHODS

Materials PEG with molecular weight 3000 g/mol was acquired from Fluka, Sigma–Aldrich (St. Loius, MO, USA). Phenolphthalein and potassium phosphates were sourced from Merck (Darmstadt, Germany). Soluble starch was acquired from Becton, Dickinson and Company (NJ, USA). The γ-CD was supplied by Sigma–Aldrich (St. Loius, MO, USA).

***B. cereus* production** The *B. cereus* culture medium was prepared with 1% (w/v) sago starch, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 0.009% (w/v) $MgSO_4$, 0.1% (w/v) K_2HPO_4 and 1% (w/v) Na_2CO_3 (autoclaved separately) (15). The inoculum was grown at 37 °C for 18 h (h) with 250 rpm continuous agitation. It was then transferred into the above mentioned medium (15), incubated at 37 °C for 30 h with 250 rpm continuous agitation speed. CGTase was harvested from supernatant centrifuged at 4000 ×*g* for 30 min.

CGTase activity analysis CGTase cyclizing activity (β-CD production) was determined employing the phenolphthalein method (15,17,18). A 50 μL sample was mixed with 750 μL substrate solutions (1% (w/v) starch in 0.05 M Tris–HCl buffer pH 8.0) and incubated at 55 °C for 10 min. Enzymatic reaction of CGTase was ended by adding 375 μL of 0.15 M NaOH followed by the addition of 100 μL 0.02% (w/v) phenolphthalein (in 5 mM Na_2CO_3) for a spectrophotometric evaluation of CGTase (550 nm). The amount of β-CD produced from the starch hydrolysis was measured using the standard curve of β-CD.

Partitioning experiments of CGTase and γ-CDs in ATPS Partition experiments were carried out at room temperature to determine the optimum ATPS for γ-CD production. Predetermined quantities of dissolved PEG, potassium phosphates, distilled water were added to reach a final total weight of 10 g ATPS containing 10% (w/w) of crude CGTase and 10% (w/w) of standard γ-CD (50 mg/mL). The established ATPSs were then shaken using vortex mixer followed by a 10 min centrifugation (4000 ×*g*). After phase separated, samples were collected for enzyme activity and γ-CD concentrations analysis.

Production of γ-CD using ATPS extractive bioconversion The ATPS extractive bioconversion of γ-CD production was carried out in a 250 mL Erlenmeyer flask. Predetermined quantities of dissolved PEG, potassium phosphates, distilled water were added to reach a final total weight of 50 g ATPS containing 5% (w/w) soluble starch and 20% (w/w) of crude CGTase. A control (without ATPS phase-forming components) was conducted for comparison. The mixture solution was kept stirring at 250 rpm and temperature controlled at 55 °C for enzymatic (CGTase) conversion of soluble starch substrate into γ-CD. Samples of top and bottom phases were collected separately at regular time intervals and heated in boiling water for duration of 5 min to denature the CGTase. The quantification of γ-CD concentration was carried out by using the reverse phase HPLC instrument.

Calculations Relative CGTase activity is expressed as the fraction of the sample's CGTase activity (U/mL) to the control.

The volume ratio, denoted by V_R, is defined as

$$V_R = \frac{V_T}{V_B} \quad (1)$$

The V_T and V_B correspond to the volume (mL) of the top and bottom phases of the ATPS respectively.

Partition coefficient of CGTase (K_{CGTase}) is given by

$$K_{CGTase} = \frac{A_T}{A_B} \quad (2)$$

where A_T and A_B represent the CGTase bioactivity (U/mL) in top and bottom phases of the ATPS respectively.

Partition coefficient of γ-CD (K_{CD}) is expressed as

$$K_{CD} = \frac{C_T}{C_B} \quad (3)$$

where C_T is the γ-CD concentration (mg/mL) in the top phase of the ATPS. The C_B represents the γ-CD concentration (mg/mL) in the bottom phase of the ATPS.

Yield of γ-CD in top phase (Y_T) is calculated using the definition

$$Y_T = \frac{1}{1 + (1/V_R * K_{CD})} * 100\% \quad (4)$$

RESULTS AND DISCUSSION

Effect of TLL on the CGTase partition and γ-CD yield Previous study showed that the relative activity of CGTase was not much affected by the different PEG molecular weights (15). In this study, the PEG with molecular weight of 3000 g/mol was selected. The TLL values of PEG/phosphates ATPSs (see Table 1) applied in the present study were referred to a previous publication (19). Table 1 shows the effects of TLL on the K_{CGTase} and the Y_T. The target of this study is to determine the