Production of Gamma-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 (Yγ = 81.88%), with CGTase partitioning in the salt-rich bottom phase (kCGTase = 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 glycosyltransferase; 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-1-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 transglycosylation 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...
investigate ways of improving the productivity and separation of $\gamma$-CD from \textit{B. cereus} CGTase \cite{15,16}. The way of optimizing the $\gamma$-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 ($V_T$) ($V_T = 0.3, 1.0, 2.0, 2.3, 4.0$ and 5.0). PEG with molecular weight 3000 g/mol was selected in this study.

**MATERIALS AND METHODS**

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

\textit{B. cereus} production \quad The \textit{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$_2$HPO$_4$ and 1\% (w/v) Na$_2$CO$_3$ (autoclaved separately) \cite{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 \cite{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 \quad CGTase catalyzing activity ($\beta$-CD production) was determined employing the phenolphthalein method \cite{15,17}. A 50 \textmu L sample was mixed with 750 \textmu L substrate solution (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 \textmu L of 0.15 M NaOH followed by the addition of 100 \textmu L 0.02\% (w/v) phenolphthalein (in 5 mM Na$_2$CO$_3$) for a spectrophotometric evaluation of CGTase (550 nm). The amount of $\beta$-CD produced from the starch hydrolysis was measured using the standard curve of $\beta$-CD.

Partitioning experiments of CGTase and $\gamma$-CDs in ATPS \quad Partition experiments were carried out at room temperature to determine the optimum ATPS for $\gamma$-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/v) of standard $\gamma$-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 $\gamma$-CD concentrations analysis.

Production of $\gamma$-CD using ATPS extractive bioconversion \quad The ATPS extractive bioconversion of $\gamma$-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 $\gamma$-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 $\gamma$-CD concentration was carried out by using the reverse phase HPLC instrument.

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

The volume ratio, denoted by $V_T$, is defined as

$$V_T = \frac{V_T}{V_B}$$

(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

$$K_{CGTase} = \frac{A_T}{A_B}$$

(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 $\gamma$-CD ($K_{CD}$) is expressed as

$$K_{CD} = \frac{C_T}{C_B}$$

(3)

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

Yield of $\gamma$-CD in top phase ($Y_T$) is calculated using the definition

$$Y_T = \frac{1}{\gamma + (1/\gamma)K_{CD}} \times 100\%$$

(4)

**RESULTS AND DISCUSSION**

Effect of TLL on the CGTase partition and $\gamma$-CD yield \quad Previous study showed that the relative activity of CGTase was not much affected by the different PEG molecular weights \cite{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 \cite{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
TLL corresponding to the system which gives the highest YT and lowest KCGTase. A higher YT indicates a higher extraction efficiency of γ-CD while a lower KCGTase suggests that more CGTase is retained in bottom phase which can be reused for the continuous extractive bioconversion process. In other words, this study on TLL allows us to optimize the KCGTase generation. Through this optimization, it was found that the optimum ATPS was obtained in PEG 3000/phosphates at TLL of 37.7% (w/w) with KCGTase of 0.56 and YT of 61.02%.

**Effect of VR on γ-CD production and separation**

The results of KCGTase and YT at different VR (VR = 0.3, 1.0, 2.0, 2.3, 4.0 and 5.0) for TLL 37.7% (w/w) are shown in Fig. 2. It was observed that the highest yield of γ-CD (YT = 81.88%) was achieved at VR = 4.0 which may be due to the higher free volume in the top phase, causing more γ-CD could be partitioned toward the top phase. The lowest γ-CD yield was observed at VR = 0.3, the reduction of top phase free volume in low VR condition may have limited the partition of the γ-CD to the PEG-rich top phase (20). The VR of 4.0 will therefore be selected for future studies.

**Effect of ATPS extractive bioconversion on γ-CD production over time**

Fig. 3 shows γ-CD concentration in the top phase of ATPS versus the extractive bioconversion process time. A 50 mL of PEG 3000/potassium phosphate at TLL of 37.7% (w/w) with VR of 4 was applied in this study. A 20% (w/w) crude CGTase and 5% (w/w) of soluble starch were added in the ATPS as described by Ng et al. (16). Reverse phase HPLC chromatogram of extractive bioconversion top phase sample with pure γ-CD standard is shown in Fig. 4A. A high peak was recorded at the retention time of 14.02 min, indicating that the retention time of γ-CD is about 14 min. In Fig. 4B, the absence of a peak in the retention time of 14 min indicates an absence of γ-CD in the initial stage of bioconversion. After 30 h of extractive bioconversion, a peak was observed at retention time of 14.43 min in Fig. 4C, which shows that γ-CD had been produced and partitioned to the top phase sample. A γ-CD concentration of 2.02 mg/mL was achieved in the ATPS top phase, which is higher than the control (1.59 mg/mL). However, 1 h (1.60 mg/mL) was suggested to be the harvest time since the γ-CD concentration has no large increment following the next h. In addition, a comparison of the CD concentration ratios (for different CDs) between the 1 h ATPS top phase sample and the control sample is shown in Table 2. The γ-CD concentration ratio of the top phase sample (19.0%) is much higher than that of the control sample (6.6%), indicating that a higher γ-CD concentration ratio product can be achieved through ATPS extractive bioconversion process.

**Repetitive batch for γ-CD production**

Repetitive batch of ATPS was performed to study the recycling of CGTase enzyme in the bottom phase. Repetitive batch of γ-CD recovery was carried out in PEG 3000/potassium phosphate ATPS at 37.7% (w/w) TLL with VR of 4. A 20% (w/w) of crude CGTase, and 5% (w/w) of soluble starch were also added into the ATPS as mentioned by Ng et al. (16). Top

**TABLE 1. Effect of the PEG and TLL upon the KCGTase and YT (%).**

<table>
<thead>
<tr>
<th>PEG molecular weight (g/mol)</th>
<th>TLL (%), w/w</th>
<th>KCGTase</th>
<th>YT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3000</td>
<td>30.7</td>
<td>0.69</td>
<td>48.68</td>
</tr>
<tr>
<td></td>
<td>34.9</td>
<td>0.58</td>
<td>54.93</td>
</tr>
<tr>
<td></td>
<td>37.7</td>
<td>0.56</td>
<td>61.02</td>
</tr>
<tr>
<td></td>
<td>40.7</td>
<td>0.54</td>
<td>59.22</td>
</tr>
</tbody>
</table>

The data obtained in this study was presented as the average of triplicate readings with an approximate inaccuracy of ±5%. Highest YT and low KCGTase were found in PEG 3000 system at 37.7% (w/w) TLL. The TLL values of PEG/phosphates ATPSs used here were referred to a previous publication (19).
FIG. 4. (A) Reverse phase HPLC chromatogram of ATPS extractive bioconversion top phase sample with pure γ-CD standard. (B) Reverse phase HPLC chromatogram of ATPS extractive bioconversion top phase sample at starting stage. (C) Reverse phase HPLC chromatogram of ATPS extractive bioconversion top phase sample after 30 h.
The ratio of CGTase enzyme was successfully recycled three times in this study. A cost saving technique which combined bioconversion. This result showed that there was a loss of CGTase activity dropped to 0.76 in the third batch of extractive phase extraction, and it can be seen that the CGTase activity exhibits relative enzyme activity of 0.95 in second batch and dropped to 0.76 in third batch of ATPS extractive bioconversion.

FIG. 5. Relative CGTase activity in each batch of soluble starch bioconversion by recycling the phase components and CGTase. The data recorded here was presented as the average of triplicate readings with an approximate inaccuracy of ±5%.

**TABLE 2.** A comparison of CDs concentration ratio (%) between ATPS top phase sample and control sample.

<table>
<thead>
<tr>
<th>CD type</th>
<th>Control (%)</th>
<th>1 h ATPS Top phase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>67.1 (12.20 mg/mL)</td>
<td>55.4 (4.67 mg/mL)</td>
</tr>
<tr>
<td>β</td>
<td>26.3 (4.78 mg/mL)</td>
<td>25.6 (2.16 mg/mL)</td>
</tr>
<tr>
<td>γ</td>
<td>6.6 (1.20 mg/mL)</td>
<td>19.0 (1.60 mg/mL)</td>
</tr>
<tr>
<td>Total</td>
<td>100.0 (18.18 mg/mL)</td>
<td>100.0 (8.43 mg/mL)</td>
</tr>
</tbody>
</table>

The CDs concentration ratio (%) in ATPS top phase sample and control sample were analyzed. The data obtained in this part of study was presented as the average of replicate readings with an approximate inaccuracy of ±5%.

**ACKNOWLEDGEMENTS**

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**References**