Extractive bioconversion of cyclodextrins by Bacillus cereus cyclodextrin glycosyltransferase in aqueous two-phase system

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Short Communication

Extractive bioconversion of cyclodextrins by *Bacillus cereus* cyclodextrin glycosyltransferase in aqueous two-phase system

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**Highlights**
- We examine extractive bioconversion in aqueous two-phase system (ATPS).
- The synthesis and recovery of cyclodextrins (CDs) is performed.
- Repetitive batch of CDs synthesis is performed.
- ATPS generates a cost-efficient and environmental friendly technique.

**Article Info**

**Abstract**

An extractive bioconversion with *Bacillus cereus* cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) in aqueous two-phase system (ATPS) was investigated for the synthesis and recovery of cyclodextrins (CDs). Optimum condition for the extractive bioconversion of CDs was achieved in ATPS consisted of 7.7% (w/w) polyethylene glycol (PEG) 20,000 and 10.3% (w/w) dextran T500 with volume ratio (\(V_R\)) of 4.0. Enzymatic conversion of starch occurred mainly in dextran-rich bottom phase whereas the product, CDs was transferred to top phase and a higher partition coefficient of CDs was achieved. Repetitive batch of CDs synthesis was employed by replenishment of the top phase components and addition of starch every 8 h. An average total CDs concentration of 13.7 mg/mL (4.77 mg/mL \(\alpha\)-CD, 5.02 mg/mL \(\beta\)-CD and 3.91 mg/mL \(\gamma\)-CD) was recovered in the top phase of PEG 20,000/dextran T500 ATPS. This study showed the effectiveness of ATPS application in extractive bioconversion of CDs synthesis with *B. cereus* CGTase.

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**1. Introduction**

Aqueous two-phase system (ATPS) is a liquid–liquid extraction technique constructed by mixing two solutions above a certain concentration as indicated by a phase diagram where formation of two immiscible aqueous phases is observed (Albertsson, 1986). ATPS is an extremely attractive approach in extractive bioconversion because its high water content and low interfacial tension properties provide a relatively mild environment for the enzymatic reaction of the biocatalysts, and thus enable the stabilization of biomaterials (Zijlstra et al., 1998). Extractive bioconversion in ATPS is a practical *in situ* product recovery technique in which product formation and separation can be integrated into a single step process by immediate removal of products from biocatalysts once they are formed (Zijlstra et al., 1998). The retention and reuse of the biocatalysts in a specific phase of the ATPS and substrate facilitates the continuous product recovery from the product-containing phase (Zijlstra et al., 1998). Extractive bioconversion in ATPS is an efficient integration process of enzymatic synthesis which potentially improves yield and productivity of the enzymatic process under a steady optimal condition as compared to other conventional extractive bioconversion (Daugulis, 1988). Besides, extractive bioconversion in ATPS can prevent the product degrada-
tation and inhibition through instant removal of the product (Freeman et al., 1993). Besides, present study has demonstrated the use of ionic liquid-based aqueous two phase system for the bioconversion process which signified the high biocompatibility of aqueous two phase system (Wu et al., 2011). In view of this, single-step ATPS was employed as a substitute to the conventional CDs recovery method to simplify the overall CDs recovery operation and to achieve higher yield of CDs by eliminating the product degradation factor.

CDs consist of six (α–CD), seven (β–CD), eight (γ–CD) or more glucopyranose units that are related by (1,4) glycosidic bonds, are products of starch degradation by CGTase through transglycosylation reaction (Martin, 2004; Szerman et al., 2007). CD molecules have the steric arrangement of glucose units featuring a hydrophilic outer surface, making CD water-soluble with a hydrophobic internal cavity (Morikaki et al., 2007). This unique structural feature of CDs enables them to form inclusion complexes with various foreign particles by partially encapsulating the compounds into their hydrophobic cavity (Loftsson and Brewer, 1996). The CDs inclusion effect alters the physiochemical properties of the guest compounds which promote the utilization of CDs in diverse industrial applications such as in pharmaceuticals, bioconversion, food and cosmetics industries. Therefore, demands for production and recovery of CDs industrially are on the rise.

Previous studies have revealed that application of ATPS in the extractive bioconversions of soluble starch from purified CGTase is feasible for CDs recovery (Chang et al., 1997; Min et al., 1996). However, there is no available information on the ATPS extractive bioconversion of CDs with crude B. cereus CGTase using sago starch as the substrate (Wang et al., 1996). In this study, repeated batch conversion of CDs by B. cereus CGTase and partition of CDs were investigated. CDs and CGTase partitioning in various ATPS based on the effects of the ATPS parameters such as phase compositions, tie-line lengths (TLLs), and Vt were studied for optimum CDs recovery. The effect of sago starch concentration on the extractive bioconversion of CDs in ATPS was also evaluated.

2. Methods

2.1. CGTase production and analytical procedures

B. cereus cultivation and enzymes production were carried out as described in previous literature (Ng et al., 2011). CGTase cyclizing activity (β–CD production) was measured using phenolphthalein method as described previously with modifications (Ng et al., 2011). α-, β- and γ-CDs were analyzed by a Shimadzu HPLC system (Liquid Chromatograph LC-10AT, Diode Array SPD-M10A, and RID 6A) equipped with a Pack Polyamine II column (250 mm × 4.6 mm, YMC Co., Ltd., Japan). Samples of CDs were eluted isocratically with water–acetonitrile (45:55) at a flow rate of 1.0 mL min⁻¹.

2.2. Enzyme stability and extractive bioconversion in ATPS

CGTase was added into each phase components solution and incubated at 55 °C for 8 h. CGTase activity was measured and the relative activity of CGTase in the phase component solution was calculated as the ratio of the CGTase activity in the phase component solution to the CGTase activity in the blank enzyme solution (0.05 M Tris–HCl buffer pH 8.0).

50% (w/w) of PEG solution, 20% (w/w) of dextran solution and 40% (w/w) of salt stock solution were prepared. Mixtures of CDs (25 mg/mL) were prepared by mixing α-, β- and γ-CD in a ratio of 1:1:1. A 10 g of ATPS was prepared in a 15 mL centrifuged tubes by adding polymer or salt stock solutions. 20% (w/w) of enzyme CGTase or mixtures of CDs was added into the ATPS. The established mixture was then shaken using vortex mixer and centrifuged at 4000 rpm for 5 min to achieve complete phase separation.

Enzymatic synthesis of CDs in ATPS was carried out in a 250 mL Erlenmeyer flask containing sago starch concentration of 6% (w/w). A 50 g ATPS and a control (reaction media without phase-forming polymers) were used for the enzymatic conversion of sago starch. 20% (w/w) of the crude CGTase was added into each ATPS. The enzymatic reaction of CGTase was performed by continuous stirring and heating at 55 °C using a magnetic stirrer. 5 mL of the aliquot was withdrawn at certain time intervals and centrifuged at 4000 rpm for 5 min for phase separation. Samples were taken from both phases of ATPS and heated in a boiling water bath for 5 min to inactivate the enzymes. The samples were then left to be cooled down at ambient temperature before subjected to analysis of CDs using HPLC. Repeated batch conversion of CDs in ATPS was accomplished by replenishing the existing product-extracting phase with the new top phase containing the identical original supply composition as the top phase of constructed ATPS (Liao et al., 1999). Same amount of fresh sago starch was added into the ATPS for continuous CDs synthesis, with the assumption that the enzymatic synthesis of CDs from starch and the substrate consumption has reached maximum. Each batch of conversion and CDs recovery required 8 h of operation.

2.3. Determination of relative CGTase activity and relative CDs concentration, Vω, partition coefficient and yield

CGTase activities and CDs concentration were calculated as the ratio of the CGTase activities (U/mL) or CDs concentrations in the ATPS to that in the control preparation (Ooi et al., 2011). Volume ratio (Vω) was defined as the ratio of the top phase volume (Vt) to that of the bottom phase volume (Vb). Partition coefficient of CGTase (KCGTase) was expressed as the ratio of CGTase cyclizing activity in the top phase (At) to the bottom phase (Ab). Partition coefficient of CDs (KCD) was expressed as the ratio of CDs concentration in the top phase (Ct) to that of the bottom phase (Cb). Yield of CDs (Yω) or recovery of CDs in the top phase of the ATPS was evaluated using Yω = 100 × [1 + (Vω × KCD)]). Overall conversion yield of CD (overall yield) is defined as the amount of CD produced (mg) per mg of starch whereas CDs productivity from each batch of conversion can also be calculated as amount of CD produced in the ATPS per hour.

3. Results and discussion

3.1. Selection of ATPS phase components for enzymatic synthesis of CDs

Effect of phase forming-components on the CGTase activity has been studied. Higher relative activity of CGTase was observed in PEG solutions as compared to EOPO solutions. The relative activity of CGTase was independent of the molecular weights of the polymers. This revealed that enzymatic reaction of CDs production remained constant even with a use of single type of polymer at different molecular weight. For the bottom phase components, CGTases activities decreased significantly with an increase of salt concentration in the solution. CGTases activities decreased significantly with an increase of salt concentration in the solution. CGTases exhibited low relative activities of approximately 1. Dextran solutions were proved to have the ability to stabilize the enzymes as reported in other literature (Chang et al., 1997; Gianfreda and Greco, 1981) and the strong ionic strength exhibited by high
concentration of salt solution will affect the reactivity and catalytically active groups of enzymes (Huddleston et al., 1994).

3.2. PEG/dextran ATPS for CDs production and recovery

Several ATPSs with different combinations of PEGs (with average molecular weight of 6000 g/mol, 8000 g/mol, 10,000 g/mol and 20,000 g/mol) and dextrans (dextran T40 and dextran T500) were tested for extractive bioconversion of CDs. CGTase and CDs will partition differentially in the ATPS as a result of the difference in sizes, surface properties and their interactions with the surrounding phase components in the ATPS. To attain an optimum condition for efficient transfer of CDs into the top phase of ATPS with exclusive CGTase partition into a specific phase of ATPS, ATPS parameters on the CDs synthesis and recovery such as average molecular weight of polymers, TLLs and Vg will be evaluated.

The phase diagrams of PEG/dextran ATPSs were referred (Diamond and Hsu, 1992). Tables 1 and 2 show the effects of TLLs on the partitioning of CGTase and CDs in the PEG/dextran T40 and PEG/dextran T500 ATPS respectively. It was observed that when the average molecular weight of the PEG increases, the \( K_{\text{CGTase}} \), decreases in both types of the PEG/dextran ATPS. High concentration of PEG will limit the partitioning of CGTase to the top phase and this is beneficial for the extractive bioconversion in ATPS where the starch hydrolysis for CDs synthesis was mainly occurred in a specific phase of the ATPS (Min et al., 1996; Zijlstra et al., 1998).

The optimum condition for CDs synthesis and recovery was achieved in PEG 20,000/dextran T500 ATPS at TLL of 26.2% (w/w) with \( K_{\text{CGTase}} \) of 0.66 (Table 1) and \( K_{\text{CD}} \) of 3.22 (Table 2). The results indicated that the efficient transfer of CDs to the top phase of the ATPS. A raise in TLL will result in a reduction in \( K_{\text{CGTase}} \) and an increase in \( K_{\text{CD}} \) due to the greater difference of hydrophobicity between both phases (Tables 1 and 2). Previous study has showed that CD would favor the dextran-rich phase rather than the PEG-rich phase (Min et al., 1996). However, high concentration of PEG and dextran in an ATPS will cause the formation of precipitant at inter-phase that complicates the recovery of CDs. Therefore, high concentrations of PEG and dextran were not applied in this study.

Table 1

<table>
<thead>
<tr>
<th>PEG mol. wt. (g mol (^{-1}))</th>
<th>TLL (% w/w)</th>
<th>( K_{\text{CGTase}} )</th>
<th>( K_{\text{CD}} )</th>
<th>( Y_{\text{T}} ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6000</td>
<td>3.7</td>
<td>1.10</td>
<td>0.83</td>
<td>45.36</td>
</tr>
<tr>
<td></td>
<td>24.3</td>
<td>1.26</td>
<td>1.04</td>
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<td></td>
<td>26.6</td>
<td>1.34</td>
<td>1.04</td>
<td>63.37</td>
</tr>
<tr>
<td>8000</td>
<td>8.8</td>
<td>1.19</td>
<td>1.04</td>
<td>53.58</td>
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<td>1.25</td>
<td>47.92</td>
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<td></td>
<td>21.2</td>
<td>1.25</td>
<td>1.25</td>
<td>47.37</td>
</tr>
<tr>
<td>10,000</td>
<td>9.3</td>
<td>1.25</td>
<td>0.93</td>
<td>65.28</td>
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<td>1.25</td>
<td>0.93</td>
<td>65.28</td>
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<td>25.8</td>
<td>1.25</td>
<td>0.93</td>
<td>65.28</td>
</tr>
<tr>
<td>20,000</td>
<td>13.2</td>
<td>1.03</td>
<td>1.03</td>
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<tr>
<td></td>
<td>25.8</td>
<td>1.03</td>
<td>1.03</td>
<td>47.37</td>
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Combination of dextran T40 with different molecular weight of PEG (6000 g/mol, 8000 g/mol, 10,000 g/mol and 20,000 g/mol) of ATPSs were constructed and \( K_{\text{CD}} \) and \( Y_{\text{T}} \) of CDs were measured. Volume ratio \( (V_v) \) was defined as the ratio of the top phase volume \( (V_A) \) to that of the bottom phase volume \( (V_B) \) where \( V_A = \frac{V_A}{V_B}, K_{\text{CGTase}} \) was expressed as the ratio of CGTase cyclizing activity in the top phase \( (A_T) \) to that of the bottom phase \( (A_B) \) where \( A_T = \frac{A_T}{A_B}, K_{\text{CD}} \) was expressed as the ratio of CDs concentration in the top phase \( (C_T) \) to that of the bottom phase \( (C_B) \) where \( C_T = \frac{C_T}{C_B}, Y_{\text{T}} \) of CDs was measured.

Table 2

<table>
<thead>
<tr>
<th>PEG mol. wt. (g mol (^{-1}))</th>
<th>TLL (% w/w)</th>
<th>( K_{\text{CGTase}} )</th>
<th>( K_{\text{CD}} )</th>
<th>( Y_{\text{T}} ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6000</td>
<td>11.2</td>
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<td>21.1</td>
<td>0.99</td>
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<td>25.9</td>
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<td>66.78</td>
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<tr>
<td>8000</td>
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<td>1.60</td>
<td>60.00</td>
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<td>1.89</td>
<td>65.40</td>
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<tr>
<td></td>
<td>25.3</td>
<td>0.87</td>
<td>2.08</td>
<td>67.53</td>
</tr>
<tr>
<td>10,000</td>
<td>15.1</td>
<td>0.91</td>
<td>1.71</td>
<td>63.10</td>
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<tr>
<td></td>
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<td>2.7</td>
<td>1.95</td>
<td>66.10</td>
</tr>
<tr>
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<td>2.12</td>
<td>2.09</td>
<td>67.64</td>
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<td>2.63</td>
<td>72.45</td>
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<td>64.66</td>
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<td>19.2</td>
<td>0.92</td>
<td>1.93</td>
<td>65.87</td>
</tr>
<tr>
<td></td>
<td>22.4</td>
<td>0.88</td>
<td>2.35</td>
<td>70.15</td>
</tr>
<tr>
<td></td>
<td>26.2</td>
<td>0.66</td>
<td>3.22</td>
<td>76.30</td>
</tr>
</tbody>
</table>

ATPSs at different \( V_v \) (0.6, 1.5, 1.71, 2.33 and 4) were tested on the synthesis and recovery of CDs and the results were shown in Table 3. The highest yield of CDs \( (Y_T = 94.81\%) \) was obtained in ATPS with \( V_v = 4.0 \). High \( V_v \) of ATPS indicates that low concentration of PEG will limit the partitioning of CGTase to the top phase and this is beneficial for the extractive bioconversion in ATPS where the starch hydrolysis for CDs synthesis was mainly occurred in a specific phase of the ATPS. A raise in TLL will result in a reduction in \( K_{\text{CGTase}} \) and an increase in \( K_{\text{CD}} \) due to the greater difference of hydrophobicity between both phases (Tables 1 and 2). Previous study has showed that CD would favor the dextran-rich phase rather than the PEG-rich phase (Min et al., 1996). However, high concentration of PEG and dextran in an ATPS will cause the formation of precipitant at inter-phase that complicates the recovery of CDs. Therefore, high concentrations of PEG and dextran were not applied in this study.

3.3. Effect of sago starch concentration on total CDs synthesis and recovery

A slight decrease of CDs concentration in top phase was observed for ATPSs containing 8–10% (w/w) of sago starch which suggest that the product inhibition have occurred in the ATPSs (Min et al., 1996). Precipitations have been observed at the inter-phase of both the ATPSs with 8% (w/w) and 10% (w/w) of sago starch respectively. Sago starch has reached its solubility limit in these ATPSs and resulted in formation of precipitants at the inter-phase of ATPS. The precipitation will hinder the transfer of CDs into the top phase of the ATPS after they are synthesised in the bottom phase of the ATPS. Besides, retention of CDs produced in the bottom phase (i.e. the enzymatic reaction site) will cause a loss of CGTase activity as a result of the degradation of CGTase by CDs. Consequently, the \( Y_T \) of CD will be affected by the substrate precipitation in ATPS. Enzymatic reaction of CGTase and transfer of CDs are not preferable in high concentration of sago starch.

3.4. Repetitive batch conversion for CDs recovery

Fig. 1 shows CDs concentration in the top phase of ATPS at regular intervals of 8 h. Total CDs concentration shows a steady
increment at the certain time period of 8 h. After 8 h of starch hydrolysis, a total CDs concentration of 13.7 mg/mL was produced with α-, β- and γ-CD in a ratio of 1.22:1.28:1 (i.e. [α-CD] = 4.77 mg/mL, [β-CD] = 5.02 mg/mL and [γ-CD] = 3.91 mg/mL respectively). Repetitive batch of CDs recovery in PEG 20,000/dextran T500 at TLL of 26.2% (w/w) with Vₚ of 4 was carried out. 20% (w/w) crude CGTase and 6% (w/w) of sago starch were applied in this study. CGTase activity exhibits an average relative value of 0.80–0.90 and shows a steady decrease in enzyme activity from batch to batch. The CGTase activity dropped to 0.50 after 4th batch of bioconversion. There is a loss of enzymes together with the removal of top phase for the CDs recovery that results in the decrease of the relative enzyme activity. The overall conversion yields for CDs are 0.2232 mg-CDs/mg-starch and the CDs productivity is 1860 mg-CDs/L/h. To recover the CDs from the PEG phase of this proposed ATPS, ultrafiltration can be applied following the ATPS procedure.

4. Conclusions

Synthesis and recovery of CDs in ATPS has been accomplished by PEG 20,000/dextran T500 ATPS at TLL of 26.2% (w/w) PEG 20,000 and 10.3% (w/w) dextran T500) with Vₚ of 4.0 and addition of 20% (w/w) CGTase and 6% (w/w) of sago starch. A total of 13.7 mg/mL of CDs was recovered in top phase of PEG 20,000/dextran ATPS after each batch of 8 h production. ATPS can be used as a cost-efficient and environmental friendly technique for the prolonged production of CGTase without involving the multiples steps of enzyme purification.

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References


