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

Eng-Poh Ng, Dr.
Short Communication

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

Hui Suan Ng\textsuperscript{a,b}, Chien Wei Ooi\textsuperscript{c}, Mohd Noriznan Mokhtar\textsuperscript{d}, Pau Loke Show\textsuperscript{d}, Arbakariya Ariff\textsuperscript{e}, Joo Shun Tan\textsuperscript{f}, Eng- Poh Ng\textsuperscript{g}, Tau Chuan Ling\textsuperscript{h,*}

\textsuperscript{a} Department of Process and Food Engineering, Faculty of Engineering, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
\textsuperscript{b} Department of Food Science and Nutrition, Faculty of Applied Sciences, UCSI University, 56000 Cheras, Kuala Lumpur, Malaysia
\textsuperscript{c} Chemical Engineering, School of Engineering, Monash University, 46150 Bandar Sunway, Selangor, Malaysia
\textsuperscript{d} Manufacturing and Industrial Processes Division, Faculty of Engineering, Centre for Food and Bioprocess Processing, University of Nottingham Malaysia Campus, Jalan Broga, Semenyih 43500, Selangor Darul Ehsan, Malaysia
\textsuperscript{e} Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
\textsuperscript{f} Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
\textsuperscript{g} School of Chemical Sciences, Universiti Sains Malaysia, Minden 11800, Malaysia
\textsuperscript{h} Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

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

Article history:
Received 2 April 2013
Received in revised form 21 May 2013
Accepted 23 May 2013
Available online 30 May 2013

Keywords:
Aqueous two-phase system
Extractive bioconversion
Cyclodextrin
Bacillus cereus
Cyclodextrin glycosyltransferase

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 \(a\)-CD, 5.02 mg/mL \(b\)-CD and 3.91 mg/mL \(c\)-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.

© 2013 Elsevier Ltd. All rights reserved.

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 \textit{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-
tion 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 α-(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 (Lofthouse 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 ATPSs based on the effects of the ATPS parameters such as phase compositions, tie-line lengths (TLLs), and Vp are 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 composition 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 original solid phase 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, Vp, 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 ATPSs to that in the control preparation (Ooi et al., 2011). Volume ratio (Vp) 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 (Yt) or recovery of CDs in the top phase of the ATPS was evaluated using Yt = 100 × (Vt × KCD). Overall conversion yield of CD (overall YCD) 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 exhibited low relative activities (i.e. relative activity of CGTase <0.4) in all the salts solutions whereas the CGTases showed high stability in both dextran solutions with 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 Vₖ 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 Kₜₐₚₑₜₑ of 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 Kₜₐₚₑₜₑ of 0.66 (Table 1) and Kₐₚₑₜₑ 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 Kₜₐₚₑₜₑ and an increase in Kₑₚₑₜₑ 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>Kₜₐₚₑₜₑ</th>
<th>Kₑₚₑₜₑ</th>
<th>Yᵣ (%)</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>8.8</td>
<td>1.19</td>
<td>1.15</td>
<td>53.49</td>
</tr>
<tr>
<td></td>
<td>24.3</td>
<td>1.26</td>
<td>1.97</td>
<td>66.33</td>
</tr>
<tr>
<td></td>
<td>26.6</td>
<td>1.04</td>
<td>1.73</td>
<td>63.37</td>
</tr>
<tr>
<td>8000</td>
<td>8.8</td>
<td>1.15</td>
<td>0.83</td>
<td>45.36</td>
</tr>
<tr>
<td></td>
<td>9.4</td>
<td>1.34</td>
<td>0.92</td>
<td>47.92</td>
</tr>
<tr>
<td></td>
<td>14.4</td>
<td>1.25</td>
<td>0.90</td>
<td>47.37</td>
</tr>
<tr>
<td></td>
<td>21.2</td>
<td>0.93</td>
<td>1.88</td>
<td>65.28</td>
</tr>
<tr>
<td>10,000</td>
<td>9.3</td>
<td>1.03</td>
<td>0.89</td>
<td>47.37</td>
</tr>
<tr>
<td></td>
<td>16.6</td>
<td>0.98</td>
<td>1.43</td>
<td>58.85</td>
</tr>
<tr>
<td></td>
<td>20.7</td>
<td>0.94</td>
<td>1.68</td>
<td>62.69</td>
</tr>
<tr>
<td></td>
<td>25.8</td>
<td>0.90</td>
<td>1.65</td>
<td>65.03</td>
</tr>
<tr>
<td>20,000</td>
<td>13.2</td>
<td>0.91</td>
<td>1.49</td>
<td>59.84</td>
</tr>
<tr>
<td></td>
<td>18.2</td>
<td>0.81</td>
<td>1.44</td>
<td>59.02</td>
</tr>
<tr>
<td></td>
<td>21.2</td>
<td>0.77</td>
<td>1.53</td>
<td>60.47</td>
</tr>
<tr>
<td></td>
<td>25.8</td>
<td>0.71</td>
<td>1.83</td>
<td>64.66</td>
</tr>
</tbody>
</table>

Combination of dextran T40 with different molecular weight of PEG (6000, 8000, 10,000 and 20,000 g/mol) of ATPSs were constructed whereas Kₑₚₑₜₑ of CDs were measured. Volume ratio (Vₑₚₑₜₑ) was defined as the ratio of the top phase volume (Vₚₑₜₑ) to that of the bottom phase volume (Vₑₚₑₜₑ) where Vₑₚₑₜₑ = Vₑₚₑₜₑ Kₑₚₑₜₑ was expressed as the ratio of CGTase catalyzing activity in the top phase (Aₑ) to the bottom phase (Aₑₑₑₑ) where CGTase activity = Vₑₚₑₜₑ and Kₑₚₑₜₑ was expressed as the ratio of CDs concentration in the top phase (Cₑₑₑₑ) to that of the bottom phase (Cₑₑₑₑ) where Cₑₑₑₑ = Vₑₚₑₜₑ Yield of CDs (Yᵣ) or recovery of CDs in the top phase of the ATPS was evaluated using Yᵣ = 100/(1 + (1/(Vₑₚₑₜₑ Kₑₚₑₜₑ))).

ATPSs at different Vₑₚₑₜₑ (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 (Yᵣ = 94.81%) was obtained in ATPS with Vₑₚₑₜₑ of 4.0. High Vₑₚₑₜₑ of ATPS indicates that low concentration of bottom phase component (i.e. dextran) was required for the ATPS formation. Thus, the cost of the ATPS for the CDs recovery could be reduced indirectly since commercial dextrans are generally expensive. Besides, high Vₑₚₑₜₑ enhances the efficiency of ATPS for CDs extraction by reducing the processing time of the ATPS. When a smaller volume of dextran (higher viscosity) dispersed in PEG (lower viscosity) continuous phase, the phase separation time will be reduced theoretically (Ooi et al., 2011).

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 ATPS 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. 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 Yᵣ 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
PEG 20,000/dextran T500 at TLL of 26.2% (w/w) for CDs production and separation was optimized by varying the \( V_b \) of ATPS. Effects of \( V_b \) on the CGTase and CDs partitioning were investigated and measured by \( K_{CGTase} \), \( K_{CD} \) and \( Y_b \) of CDs exhibited in the ATPS. Volume ratio \( (V_b) \) was defined as the ratio of the top phase volume \( (V_T) \) to that of the bottom phase volume \( (V_B) \) where \( V_b = \frac{V_T}{V_B} \). \( K_{CGTase} \) was expressed as the ratio of CGTase cycling activity in the top phase \( (A_T) \) to the bottom phase \( (A_B) \) where CGTase activity \( \frac{A_T}{A_B} \) and \( K_{CD} \) was expressed as the ratio of CDs concentration in the top phase \( (C_T) \) to that of the bottom phase \( (C_B) \) where \( K_{CD} = \frac{C_T}{C_B} \). Yield of CDs \( (Y_b) \) or recovery of CDs in the top phase of the ATPS was evaluated using \( Y_b = 100/ \left[ 1 + (1/(V_b + K_{CD})) \right] \).

**Table 3**

<table>
<thead>
<tr>
<th>( V_b )</th>
<th>( K_{CGTase} )</th>
<th>( K_{CD} )</th>
<th>( Y_b ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.60</td>
<td>0.87</td>
<td>4.56</td>
<td>87.44</td>
</tr>
<tr>
<td>1.50</td>
<td>0.74</td>
<td>5.20</td>
<td>91.23</td>
</tr>
<tr>
<td>1.71</td>
<td>0.71</td>
<td>6.08</td>
<td>91.47</td>
</tr>
<tr>
<td>2.33</td>
<td>0.69</td>
<td>7.16</td>
<td>92.38</td>
</tr>
<tr>
<td>4.00</td>
<td>0.40</td>
<td>11.60</td>
<td>94.81</td>
</tr>
</tbody>
</table>

PEG 20,000/dextran T500 at TLL of 26.2% (w/w) for CDs production and separation was optimized by varying the \( V_b \) of ATPS. Effects of \( V_b \) on the CGTase and CDs partitioning were investigated and measured by \( K_{CGTase} \), \( K_{CD} \) and \( Y_b \) of CDs exhibited in the ATPS. Volume ratio \( (V_b) \) was defined as the ratio of the top phase volume \( (V_T) \) to that of the bottom phase volume \( (V_B) \) where \( V_b = \frac{V_T}{V_B} \). \( K_{CGTase} \) was expressed as the ratio of CGTase cycling activity in the top phase \( (A_T) \) to the bottom phase \( (A_B) \) where CGTase activity \( \frac{A_T}{A_B} \) and \( K_{CD} \) was expressed as the ratio of CDs concentration in the top phase \( (C_T) \) to that of the bottom phase \( (C_B) \) where \( K_{CD} = \frac{C_T}{C_B} \). Yield of CDs \( (Y_b) \) or recovery of CDs in the top phase of the ATPS was evaluated using \( Y_b = 100/ \left[ 1 + (1/(V_b + K_{CD})) \right] \).

**4. Conclusions**

Syntesis and recovery of CDs in ATPS has been accomplished by PEG 20,000/dextran T500 ATPS at TLL of 26.2 [which is equivalent to 7.7% (w/w) PEG 20,000 and 10.3% (w/w) dextran T500] with \( V_b \) 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 multiplies steps of enzyme purification.

**Acknowledgements**

This work is supported financially by eScience fund (02-01-03-SF0649) from the Ministry of Science, Technology and Innovation (MOSTI), Malaysia.

**References**


