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A Novel Method for Continuous Production of Cyclodextrins Using an Immobilized Enzyme System

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ABSTRACT

Selected specific α-amylase and glucoamylase enzymes were immobilized and combined with immobilized cyclodextrin glycosyltransferase to continuously produce cyclodextrin (CD). The liquefied starch was cyclized to CD by ICGTase and then treated by immobilized α-amylase and glucoamylase to contain CD and glucose only. β-CD and soluble CD (α- and γ-CD) in the final CD solution could be easily separated. The pH–activity curve, temperature–activity curve, batch re-usability and continuous operation stability of immobilized enzymes were discussed. The continuous production of CD by an immobilized enzyme system was also reported. The optimal conditions for using immobilized α-amylase and glucoamylase simultaneously were 40°C and pH 4·5 adjusted by 1 mol dm^{-3} HCl/NaOH. A 70% yield of CD could be obtained from 1% (w/v) of liquefied starch under continuous operation at 0·055 h^{-1} space velocity, and almost all the oligosaccharides (≈98%) were converted to glucose. In this study, the separation of α-CD, γ-CD and glucose, using organic solvent from the final product after precipitating β-CD, was also investigated.

Key words: cyclodextrin, immobilized enzymes, α-amylase, glucoamylase, cyclodextrin glycosyltransferase.

1 INTRODUCTION

Cyclodextrin (CD) was first produced on an industrial scale in 1976.[1-4] The traditional production method includes three procedures; first, the liquefication reaction of starch; second, the CD formation reaction from liquefied starch; and

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third, separation and purification of CD. In previous papers, CD was precipitated and separated by adding organic solvent,[4] but using this method it was difficult to handle high concentrations and the highly viscous liquid. In recent years, membrane technology has been rapidly developed and applied in CD production to separate CD from dextrins,[5-8] but there are still some problems to overcome, especially when the concentration and viscosity of CD solutions are high. In this paper, a method has been established by using specific immobilized α-amylase and glucoamylase to hydrolyze all the linear polysaccharides to glucose, and reserve all CD during the production of CD by immobilized cyclodextrin glycosyltransferase (ICGTase). As a result, the final products contained only α-, β-, γ-CD and glucose. α-Amylase was used to enhance the hydrolysis of polysaccharides by glucoamylase. The immobilization of α-amylase[9,10] and glucoamylase[9-13] have already been extensively studied. In this study the immobilization of these two enzymes was based on consideration of the cost of specific enzymes and the prospect for continuous operation. The properties of immobilized enzymes and optimal reaction conditions for production of CD are discussed.

Treatment by the immobilized enzyme system gave a final CD syrup containing only CD and glucose. β-CD could be crystallized after being concentrated by evaporation. The remaining water-soluble α- and γ-CD could be recovered by using trichloroethylene, and a glucose by-product could also be obtained. In ordinary production of β-CD, the residual liquid after crystallization of β-CD is generally not further treated and usually used as a food additive. The remaining valuable α- and γ-CD are difficult to separate, due to their higher solubility, and are wasted. By using the immobilized enzyme method developed in this study, not only was pure β-CD obtained, but also valuable α- and γ-CD were recovered.

In a previous paper, the production of CD from starch by ICGTase was investigated.[14] In this study, immobilized α-amylase and glucoamylase were used in combination with ICGTase to produce CD from liquefied starch continuously. The separation of water-soluble α- and γ-CD from final products using trichloroethylene is also reported.

2 MATERIALS AND METHODS

2.1 Materials

CGTase, from alkaline *Bacillus* sp. (33 000 u g^{-1} of activity) was purchased from Meito Sangyo Co. (Tokyo, Japan). Chitin and α-amylases from *Bacillus subtilis*, porcine pancreas, barley malt, *Aspergillus oryzae*, and *Bacillus lichenigormis* were all purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Glucoamylase AMG 300L was obtained from Novo Co. (Bagsvaerd, Denmark), *Rhizopus* genus and *Aspergillus niger* were purchased from Sigma and *Rhizopus niveus* was purchased from Seikagaku Kogyo Co. (Tokyo, Japan).

2.2 Immobilization of CGTase and preparation of CD liquid

The immobilization of CGTase was carried out using chitosan (prepared by the treatment of chitin) as the support, and liquefied starch was prepared from 5% (w/v)

of potato starch by CGTase.[5] Liquefied potato starch (1 dm^3 of 5 wt %) was reacted with 20 g of ICGTase (containing 166 mg of CGTase) at pH 8·5 and 55°C for 24 h to give CD solution I. The liquid was treated at 100°C for 10 min and subjected to HPLC analysis to determine the concentration of CD.

2.3 Selection and immobilization of α-amylase and the hydrolysis of polysaccharides in CD formation liquid

α-Amylases from five sources, viz. *Bacillus subtilis*, porcine pancreas, barley malt, *Aspergillus oryzae* and *Bacillus lichenigormis* were each reacted with 5 % (w/v) of CD solution I at pH 6·0 and 55°C for 21 h. Samples were taken to determine the viscosity and the amount of residual CD in the reaction solution and to compare the effect of various enzymes. Two supports were used to immobilize the selected α-amylase. (1) Calcium-alginate: 2 g of α-amylase was suspended in 40 cm^3 water and 2·5 wt % of calcium-alginate was added and stirred. The mixture was passed through a small peristaltic pump and dropped into a cold, stirred 0·1 mol dm^{-3} calcium chloride solution giving gel beads of approximately 2 mm diameter. (2) Chitosan: the procedures were the same as described in a previous paper.[14]

2.4 Selection and immobilization of glucoamylase and glucoamylation of CD liquid

Glucoamylases from four sources viz. *Aspergillus niger*, *Rhizopus niveus*, *Rhizopus* genus and Novo AMG 300L (a commercial product) were each reacted with 5 % (w/v) CD solution II (already treated by immobilized α-amylase) at pH 5·0 and 40°C and samples taken periodically for measurement of glucose formed and residual CD. On this basis the most effective enzyme was selected to be immobilized in calcium-alginate gel as described as above.

2.5 Continuous production of CD

Immobilized CGTase, α-amylase and glucoamylase were packed into a glass tube (2 cm × 20 cm), then liquefied starch (1–5 % w/v) was continuously passed through the immobilized enzyme reactors to produce CD. The overall flow chart is shown in Fig. 1. The amount of CD after reactor (A) and (C) and the amount of glucose after reactor (C) were determined to calculate the conversion of CD and glucose.

2.6 Separation of α-, β-, γ-CD and glucose

A portion of 500 cm^3 of the final products (CD solution III) for the continuous production of CD from 5 % (w/v) starch was decolorised and taken to separate the α-, β-, γ-CD and glucose. Firstly, the mixture was mixed with the organic solvent trichloroethylene (100 cm^3) to extract CD, a white precipitate was formed and collected by filtration and the procedures of precipitation with trichloroethylene were repeated twice. The final liquid was distilled to dryness and analyzed by HPLC to determine the contents. The residual liquid was concentrated to dryness to give glucose. In the second method, the CD-containing solution was concentrated to 50 cm^3 and allowed to stand at 4°C overnight to crystallize. Crystallized β-CD was separated by filtration and the remaining soluble CD was then extracted by trichloroethylene.

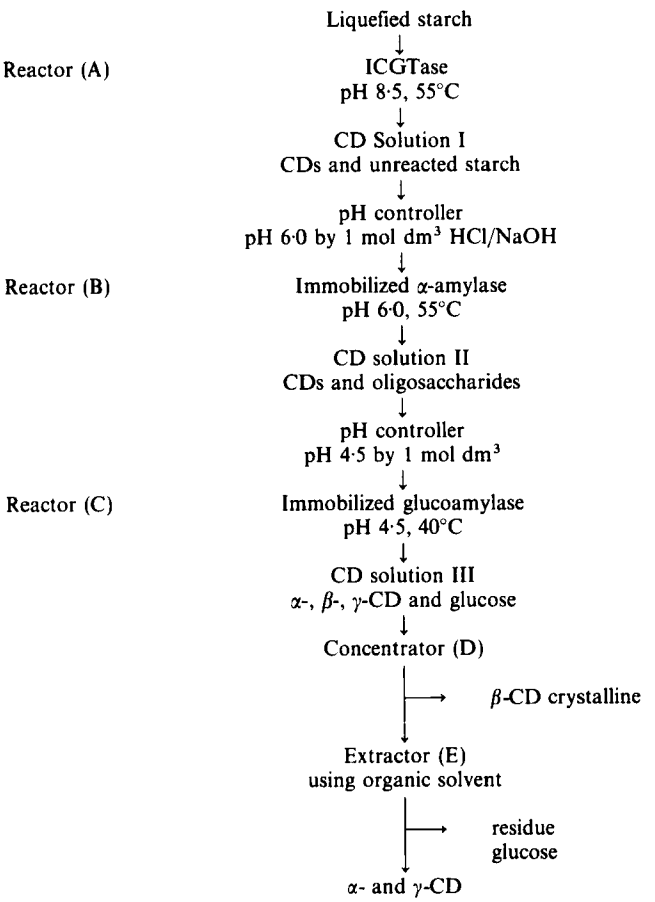

Fig. 1. Flow chart for production of α-, β- and γ-CD from starch using immobilized enzymes.

2.7 Analytical methods

The quantitation of reducing sugar was by the 3,5-dinitrosalicyclic acid method.[15] Glucose formed during the glucoamylation reaction with immobilized glucoamylase and the CD concentration in the CD solution were assayed by HPLC, the instrument was a Type LC-4A (Shimadzu Co., Kyoto, Japan), the column used was a μBondapak carbohydrate (3·9 cm × 30 cm), the solvent was a mixture of acetonitrile and water (72:28% v/v), the flow rate, 1·0 cm^3 min^{-1}, and operating pressure 80–100 kg cm^{-2}. The viscosity (η_{sp} c^{-1}) of the reaction solution during the hydrolysis of CD solution with immobilized α-amylase was determined by a Cannon Fenske viscometer using pure water as the standard.

3 RESULTS AND DISCUSSION

3.1 Continuous CD production

A suitable concentration of liquefied starch was passed through the ICGTase reactor (A) to give CD solution I (containing cyclodextrins and linear dextrins—

starch with a lower molecular weight); after adjusting the pH the solution was passed through the immobilized α-amylase reactor (B) to give CD solution II in which the linear dextrin was hydrolyzed, so reducing the viscosity of the CD solution without degrading the CD itself. The pH of the CD liquid was adjusted again and passed into the immobilized glucoamylase reactor (C) to convert the oligosaccharides, with the exception of CD, to glucose. The reactors (B) and (C) could also be combined to form one reactor. The final CD liquid (CD solution III) contained only CD (including α-, β-, and γ-CD) and glucose, β-CD could then be precipitated in concentrator (D). The residual liquid flowed into the extractor (E) to form an inclusion compound of water-soluble α- and γ-CD with organic solvent and the final residual liquid was distilled to dryness to give glucose. The CD liquid obtained from reactor (A) was treated at 100°C for 10 min when necessary to prevent the decomposition of CD with reducing sugars (such as glucose and maltose) by CGTase leakage into solution during the following processes in reactor (B) and (C). The overall process for continuous operation is shown in Fig. 1.

3.2 Preparation and properties of immobilized enzymes

3.2.1 Immobilization of CGTase and preparation of CD liquid

CGTase was immobilized on chitosan using glutaraldehyde.[14] Liquefied starch, 5% (w/v), was reacted with immobilized CGTase at pH 8·5 and 55°C for 24 h to yield CD. The resulting CD solution contained 53% (w/v) of CD (α-CD 31·4%, β-CD 6·2% and γ-CD 15·4%) and 46·4% (w/v) of dextrins. When necessary the CD solution was heated at 100°C for 10 min to deactivate the remaining enzyme in solution, so preventing the decomposition of CD.

3.2.2 Selection and immobilization of α-amylase

α-Amylases hydrolyze the viscous starch molecule to form oligosaccharides with lower molecular weight and decrease the viscosity. The hydrolysis efficiency of starch and the degradation of CD by different α-amylases depended on their substrate specificities. The purpose of selecting α-amylase was to conserve all the CD but to hydrolyze as much of the unreacted starch as possible during the hydrolysis of CD solution. Table 1 illustrates the results of the hydrolysis of CD

TABLE 1
Reaction of CD Solution I with Various α-Amylases

Sources of α-amylase	*Total cyclodextrins*		
	Initial[a] ($g\ cm^{-3}$)	*Final* ($g\ cm^{-3}$)	*Remaining* (%)
Bacillus subtilis	26·5	25·6	96·8
Porcine pancreas	26·5	16·1	60·9
Barley malt	26·5	18·1	68·3
Aspergillus oryzae	26·5	5·2	19·5
Bacillus lichenigormis	26·5	20·2	76·3

The initial CD solution contains 3·1, 15·7 and 7·7 g dm^{-3} of α-, β-, and γ-CD respectively.

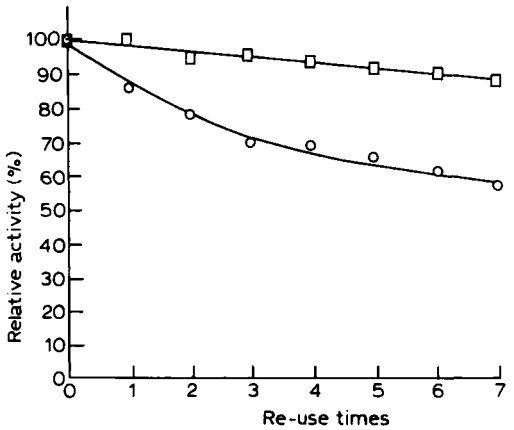

Fig. 2. Batch re-usability of immobilized α-amylase. Each reaction was carried out at pH 6·0, 55°C for 23 h for α-amylase immobilized on (□) chitosan and (○) calcium-alginate gel.

solution I by various α-amylases, 10 cm^3 of 5% (w/v) CD solution I reacted with α-amylase from various sources for 21 h at pH 6·0 and 55°C, 1200 u of each enzyme was added. α-Amylase from *Bacillus subtilis* was found to preserve almost all the CD (≈97%) and thus was selected for immobilization. In this study, chitosan and calcium alginate were used as matrix for immobilized α-amylase. The batch re-usability of the prepared immobilized enzymes in hydrolyzing CD solution is shown in Fig. 2. The re-usability of α-amylase immobilized onto chitosan was superior to that entrapped in calcium-alginate gel. This might be because enzyme chemically bound to chitosan by glutaraldehyde linkage is less prone to leaching than that immobilized in calcium-alginate gel.

3.2.3 Selection and immobilization of glucoamylase

Glucoamylases hydrolyze oligosaccharides and polysaccharides to form glucose. The effects of glucoamylases from various sources on CD solution II is shown in Fig. 3. The glucoamylase derived from *Rhizopus niveus* gave the highest level of residual CD and also gave almost maximum hydrolysis (≈96%) of the saccharides to glucose and was therefore selected for subsequent studies with enzyme immobilized in calcium-alginate gel beads. Leakage of enzyme from the beads on re-use was found to be significant. Thus, after seven repeated reaction cycles only 60% of the initial activity was retained.

3.2.4 Properties of immobilized enzymes

ICGTase has been described previously.[14] Figure 4 shows that for the reaction studied here the pH optima of both free and immobilized α-amylase were between pH 6·0 and 6·5, but the immobilized α-amylase shows a higher relative activity than the free enzyme at more alkaline pH value (pH > 8·0). The immobilized glucoamylase has an optimal pH range of between pH 4·0 and 5·5, which is broader than that of the free enzyme. The optimal reaction temperature for both free and immobilized α-amylase in hydrolyzing CD solution is about 75°C, and 35–45°C for

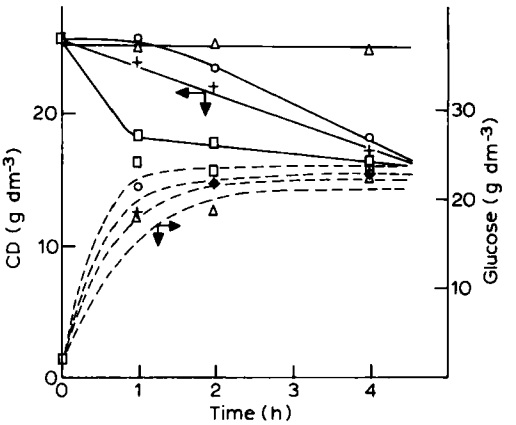

Fig. 3. Reaction of CD solution I with glucoamylases from various sources. Substrate (10 cm^3) was reacted with 76 u of enzyme derived from (○) *Aspergillus niger*; (△) *Rhizopus niveus*; (+) *Rhizopus* genus; (□) Novo AMG 300L, at pH 5·0 and 40°C.

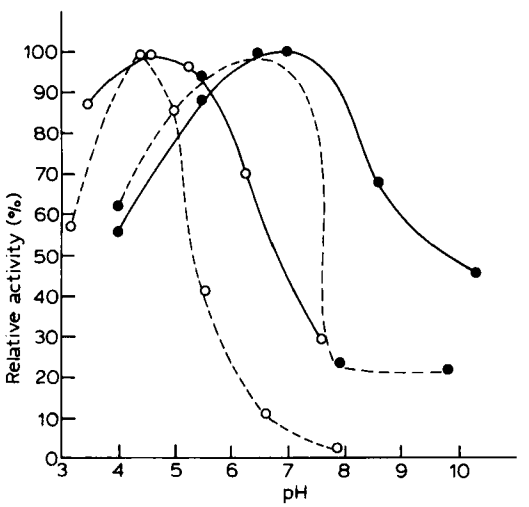

Fig. 4. pH-activity curve of immobilized α-amylase and glucoamylase. (●) α-amylase; (○) glucoamylase; (——) free enzymes; (– – –) immobilized enzymes.

both free and immobilized glucoamylase. The continuous reaction of immobilized α-amylase (pH 6·0, 55°C) and glucoamylase (pH 4·5, 40°C) with substrate is illustrated in Fig. 5. This shows that for both enzymes 90% of initial activity remained after 35 h of continuous operation indicating only slight loss of enzyme from the matrix.

The viscosity and pH changes during the reaction of immobilized α-amylase with CD solution at various initial pH values are shown in Fig. 6. It was found that using 1 mol dm^{-3} HCl/NaOH to adjust the initial pH value gave the same pH control effect as phosphate buffer, and thus immobilized α-amylase could hydrolyze CD solution at the optimum pH. Figure 7 shows that 1 mol dm^{-3} HCl/NaOH was able

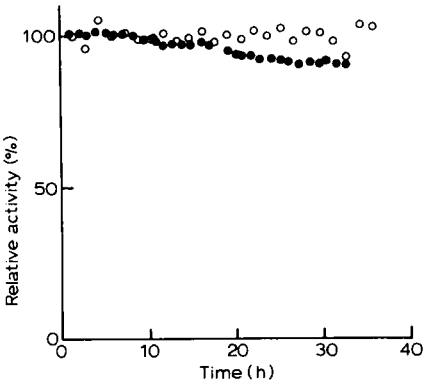

Fig. 5. Continuous operation stability of immobilized α-amylase and glucoamylase. Substrate (CD solution I) was passed through (○) immobilized α-amylase at 10 $cm^3 h^{-1}$, pH 6·0 and 55°C; (●) immobilized glucoamylase at 3 $cm^3 h^{-1}$, pH 4·5 and 40°C.

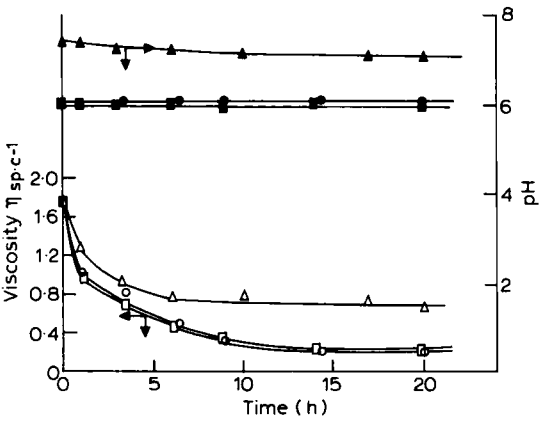

Fig. 6. Changes in viscosity (open symbols) and pH (solid symbols) during the reaction of CD solution I with immobilized α-amylase at various pH conditions. Initial pH (▲, △) 7·4, without any adjustment; (●, ○) 6·2, adjusted by 1 mol dm^{-3} HCl/NaOH; (■, □) 6·0, adjusted by 0·01 mol dm^{-3} phosphate buffer.

to maintain the optimal pH at 4·5 for immobilized glucoamylase to convert saccharides to glucose. The advantage of using HCl/NaOH to replace buffer is that the organic salt content of the product is minimized and the separation made easier. The optimal pH and temperature for a mixture of immobilized α-amylase and glucoamylase reacting with CD solution is pH 4·5 and 40°C respectively.

3.2.5 Continuous production of CD

The overall process for continuous production of CD is shown in Fig. 1. Each reactor (A), (B) and (C) composed a glass tube of 60 cm in length and 2 cm in diameter. The liquefied starch was continuously pumped into the immobilized enzyme reactors, and samples were taken in the outlet of reactors (A) and (C) to determine the conversion of CD and glucose. Figure 8 shows that the conversions of

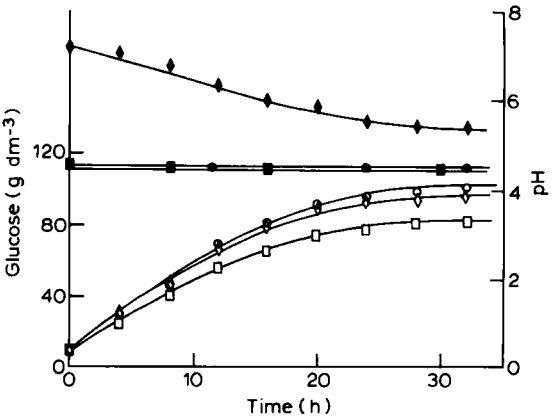

Fig. 7. The formation curve of glucose (open symbols) and pH change (solid symbols) during the reaction of CD solution II with immobilized glucoamylase at various pH conditions. Initial pH (◆, ◇) 7·1, without adjustment; (●, ○) 4·5, adjusted by 0·01 mol dm^{-3} acetate buffer; (■, □) 4·5, adjusted by 0·01 mol dm^{-3} acetate buffer. Reaction temperature = 40°C.

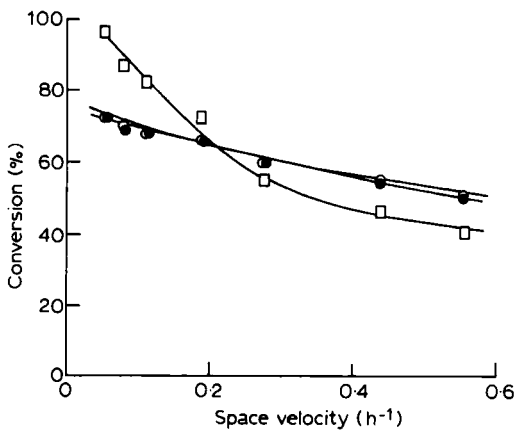

Fig. 8. Effects of space velocity on the conversion of CD and glucose by immobilized enzyme system at continuous operation. Substrate 1% w/v liquefied starch. Conversion of CD (○) after reactor (A); (●) after reactor (C). (□) Conversion of glucose after reactor (C). Reaction conditions are shown in Fig. 1.

both CD and glucose increased as the space velocity (SV, as defined in eqn (1))

$$\text{SV } (h^{-1}) = \frac{\text{pumped rate of substrate } (cm^3\ h^{-1})}{\text{space volume of reactor } (cm^3)} \quad (1)$$

at which substrate was pumped decreased. It was also observed that the concentration of CD was the same in the eluates of both reactors (A) and (C) indicating that the amount of CD produced by ICGTase in reactor (A) did not change after reaction with immobilized α-amylase and glucoamylase. As a result, a 70% conversion of CD was obtained and 98% of the remaining polysaccharides were converted to glucose from 1% (w/v) of liquefied starch at SV = 0·055 (h^{-1}) for

TABLE 2
Separation of α-CD, β-CD, γ-CD and Glucose

	Weight (g)	*Content (g)*				
		α-	*β-*	*γ-*	*Total CD*	*Glucose*
Initial CD liquid	—	1·55	7·85	3·85	13·25	11·6
Method (1)						
Trichloroethylene precipitate	12·99	1·50	7·72	3·75	12·97	—
Residue (solid)	11·90	0·05	0·13	0·10	0·28	11·6
Method (2)						
1. Concentrated β-CD crystalline	7·48	—	7·48	—	7·48	—
Residual liquid	—	1·55	0·37	3·85	5·77	11·5
2. Trichloroethylene precipitation						
First precipitate	5·12	1·30	0·34	3·37	5·01	—
Second precipitate	0·68	0·21	0·01	0·44	0·66	—
Residual solid	11·84	0·04	0·02	0·04	0·10	11·5

continuous operation of the immobilized enzyme reactor system. During the continuous operation, the yield of CD was affected by starch concentration, temperature and pH value, rate of substrate and reactor geometry. In order to reach the same conversion of CD at higher starch concentration, the reactor size should be increased or the flow rate decreased.

3.2.6 Separation of α-, β-, γ-CD and glucose

The contents of the initial mixture and the products of each separation procedure were analyzed by HPLC and the results are summarized in Table 2. In the first method, trichloroethylene extraction gave 12·97 g of a mixture of α-, β-, and γ-CD (1·50 g, 7·72 g and 3·75 g respectively) from 500 cm^3 of 5% (w/v) CD solution, and 11·9 g of residue containing 11·5 g glucose. The second method yielded 7·48 g of β-CD crystalline, and twice trichloroethylene extractions gave 5·8 g of soluble CD mixture containing α-CD (1·51 g), β-CD (3·81 g) and γ-CD (0·35 g) and 11·84 g of final residue containing 11·5 g glucose.

4 CONCLUSION

An immobilized enzyme reactor system which includes ICGTase, α-amylase and glucoamylase has been used for the continuous production of cyclodextrins. α-Amylase from *Bacillus subtilis* and glucoamylase from *Rhizopus niveus* gave the best results for the hydrolysis of polysaccharides and for conserving the CD in reaction mixtures. The final product could be concentrated and extracted by organic solvent to separate α-CD, β-CD, γ-CD and glucose.

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