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n-Octyl β-D-glucoside synthesis through β-glucosidase catalyzed condensation of glucose and *n*-octanol in a heterogeneous system with high glucose concentration

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Abstract

β-Glucosidase-catalyzed synthesis of *n*-octyl β-D-glucoside through condensation was performed by mixing glucose and octanol saturated with acetate buffer at 50°C. The highest yield of synthesis, 28% based on glucose, was obtained at the total glucose concentration of 0.1 mol/l. At higher glucose concentrations, the product concentration increased but the yield decreased. The stability of the enzyme in the system was so high that, even in the free form, it could be used repeatedly for synthesis without a significant loss in its activity. The overall yield of the four consecutive batch syntheses was 58% when the initial glucose concentration was adjusted to 0.1 mol/l for each batch.

Keywords: Octyl glucoside; β-D-Glucosidase; Low-water system; Organic solvents; Biosurfactant

1. Introduction

It has been shown that many hydrolytic enzymes exhibit their catalytic activities in water–organic two-phase system or in organic medium with low water content [1]. This enabled to use the enzymes in synthesis of organic compounds through reverse hydrolysis (condensation) or transfer reaction. Due to the regio- and stereospecificity of enzyme catalysis, the reaction allows the product of interest to be obtained in very high purity. The enzymatic synthesis of organic compounds has thus been an active area of research, and a recent review of the application of the enzymes to the synthesis of surface-active compound emphasized the achievements in this field [2]. The products have sometimes been called biosurfactants or biological surfactants.

Alkyl glucosides, a group of nonionic surfactants, are widely used in many different industries; they are used, for example, in cosmetics, pharmaceuticals, detergents etc. Their chemical syntheses are well developed [3] and used at the commercial level. However, the processes are complicated by the presence of identically reactive hydroxyl groups, and by the toxicity of some catalysts used. It is, therefore, desirable to find an alternative and economical procedure for the synthesis that can overcome these problems. A number of papers on the enzymatic

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syntheses of alkyl glucosides have been reported [4–12]: they include the synthesis of octyl β-D-glucoside [4–6,8–11]. Usually, these syntheses were performed in a low-water system in which the reaction could be shifted towards synthesis. It was found that a commercially available glucosylhydrolase, almond β-glucosidase, was effective as a biocatalyst for the synthesis of these substances through transglycosylation or condensation. For the former, the substrates used were phenyl glucoside, alkyl glucoside with a short alkyl chain and disaccharide. A recent study [10] showed that two glycosides, octyl glucoside and octyl galactoside, could be synthesized simultaneously from lactose in the presence of a combination of enzymes. β-Glucosidase from almond was shown to be active for condensation of glucose with a broad range of primary [4–11], secondary [11] or aromatic alcohols [12]. The suitable system for the synthesis seems to be the water–organic two-phase system since the substrates, glucose and alcohol, have opposite polarity; i.e. the former is hydrophilic while the latter is hydrophobic. In this system, the reaction proceeds in the aqueous phase where the enzyme, the polar substrate and a small amount of apolar substrate are present, and the product partitions to the organic (alcohol) phase. The equilibrium yield of the alkyl glucoside in the system is dependent on the substrate concentrations, partition coefficient of the product, and the volume ratio of the two phases [11]. In some studies, β-glucosidase was used in an immobilized form on a synthetic polymer such as Amberlite XAD 4 [5,6,9], and it was shown that this enzyme preparation is highly stable in the system.

However, the product yields attained in such studies were not high. In this paper, we describe a reaction system to increase the yield of alkyl glucoside. The synthesis of octyl glucoside was investigated as a model reaction. Octyl glucoside was produced through a condensation of D-glucose and *n*-octanol by β-glucosidase in a system where solid glucose was added to a buffer-saturated octanol to form a separate phase including glucose and the enzyme. An additional advantage of this system is the high stability of the enzyme even without immobilization, which made it possible to reuse the enzyme for repeated rounds of synthesis.

2. Materials and methods

2.1. Materials

β-D-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) from sweet almond was obtained from Toyobo, Osaka, Japan, and was used without further purification. *n*-Octyl β-D-glucoside, used as a standard in HPLC analysis, was purchased from Dojin Chemical Institute, Kumamoto, Japan. All other reagents and organic solvents were of the highest grade available commercially from Wako Pure Chemical Industries, Japan. *n*-Octanol was pre-equilibrated before use by mixing at room temperature with 0.2 mol/l acetate buffer, pH 5.5, and then left to stand overnight. The mixture was then centrifuged to obtain good separation. The upper phase, buffer-saturated octanol, contained 4.0% (v/v) of water. This phase was used as a reaction medium for octyl glucoside synthesis.

2.2. Reduction of water content in the octanol phase by addition of glucose

Four ml of buffer-saturated octanol was incubated with anhydrous glucose but without enzyme, and followed by mixing at 50°C with vigorous stirring overnight to ensure equilibrium. The water content of the octanol phase was determined by Karl Fischer titration.

2.3. n-Octyl β-D-glucoside synthesis

Pre-equilibrated buffer-saturated octanol was added to a glass bottle containing glucose and enzyme, and the reaction was carried out at 50°C with vigorous stirring. At appropriate intervals, a portion of the octanol phase was

sampled and *n*-octyl β-D-glucoside produced was analyzed by HPLC.

2.4. Enzyme reuse

Synthesis was performed at a glucose concentration of 0.1 mol/l or 0.35 mol/l in 20 ml of buffer-saturated octanol. The period for each cycle of synthesis was 6 days. In the cases where the initial glucose concentration was 0.1 mol/l, at the end of incubation the reaction mixture was centrifuged to separate the phases. The upper octanol phase containing the product was removed with a Pasteur pipette, and fresh buffer-saturated octanol and the same amount of glucose as the synthesized product was added. Then, the reaction was run again under the same conditions. Synthesis was repeated for a further two runs. Similar experiments were performed when the initial glucose concentration was 0.35 mol/l, except that glucose was not added to restore the concentration for subsequent synthesis.

2.5. HPLC analysis

The product, octyl glucoside, was analyzed by high-performance liquid chromatography as described previously [13]. The liquid chromatograph (Shimadzu LC-6A, Kyoto, Japan) was equipped with a refractometer (RI SE-51, Showa Denko, Tokyo, Japan), and separation was performed at room temperature on a column (4.6 mm $\phi \times 250$ mm) packed with TMP-10P gel (porous trimethylolpropane trimethacrylate gel), supplied by Japan Organo, Tokyo, Japan. The eluent was a 2:1 methanol–water mixture (v/v), at a flow rate of 1 ml/min. The concentration of octyl glucoside was determined from the peak area using a calibration curve prepared using a commercial preparation.

2.6. Identification of the product

The product purified by HPLC was analyzed by ^{1}H NMR and FAB–MS. To confirm the position of alkylation, the product was acetylated using acetic anhydride and pyridine to yield the tetraacetate. The chemical shift of the anomeric proton did not change, while significant down field shift was observed in the protons at 2, 3, 4 and 6. This indicates that the position of alkylation is 1. Product: ^{1}H NMR δ (2 mg/0.5 ml D_2O, 300 K, ref. DSS) ppm: 0.87 (3H, t, $J = 6.8$ Hz, H_3-8′), 1.25–1.40 (10H, m, H_2-3′–7′), 1.63 (2H, m, H_2-2′), 3.26 (1H, dd, $J = 9.0$, 8.0 Hz, H-2), 3.38 (1H, t, $J = 9.3$ Hz, H-4), 3.44 (1H, m, H-5), 3.49 (1H, t, $J = 9.2$ Hz, H-3), 3.68 (1H, m, H-1′a), 3.72 (1H, dd, $J = 12.3$, 5.9 Hz, H-6a), 3.92 (2H, m, H-1′b, H-6b), 4.45 (1H, d, $J = 8.0$ Hz, H-1). Acetylated product: ^{1}H NMR δ (1 mg/0.5 ml $CDCl_3$, 300 K, ref. TMS) ppm: 0.88 (3H, t, $J = 6.9$ Hz, H_3-8′) 1.25–1.40 (10H, m, H_2-3′–7′). 1.57 (2H, m, H_2-2′), 2.00 (3H, s, Ac), 2.02 (3H, s, Ac), 2.04 (3H, s, Ac), 2.08 (3H, s, Ac), 3.47 (1H, dt, $J = 9.6$, 6.8 Hz, H-1′a), 3.69 (1H, ddd, $J = 9.9$, 4.7, 2.3 Hz, H-5), 3.86 (1H, dt, $J = 9.6$, 6.4 Hz, H-1′b), 4.14 (1H, dd, $J = 12.3$, 2.3 Hz, H-6a), 4.26 (1H, dd, $J = 12.3$, 4.7 Hz, H-6b), 4.49 (1H, d, $J = 8.0$ Hz, H-1), 4.98 (1H, dd, $J = 9.6$, 8.0 Hz, H-2), 5.08 (1H, t, $J = 9.7$ Hz, H-4), 5.20 (1H, t, $J = 9.5$ Hz, H-3). Product: FAB-MS (glycerol as a matrix) m/z 293 (MH^+).

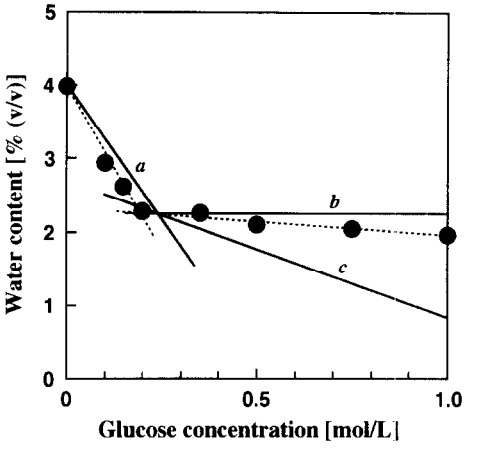

Fig. 1. Relationship between the water content in the octanol phase and the overall glucose concentration in the system at 50°C. The system contains no enzyme. The broken lines are drawn empirically. See text for descriptions of the solid lines a, b and c.

3. Results and discussion

3.1. Reaction system

When solid glucose was mixed with buffer-saturated octanol at 50°C, it was gradually dissolved by the water from the octanol phase. Simultaneously, a separate aqueous phase, containing glucose, water and a small amount of octanol, was formed. The water content in the octanol phase decreased as the amount of glucose increased. Fig. 1 shows the relationship between the water content in the octanol phase and the overall glucose concentration in the system. As shown in the figure, the plots could be separated into two regions, region I with the overall glucose concentration $\bar{C}_G$ less than 0.2 mol/l and region II with $\bar{C}_G > 0.2$ mol/l, and a straight line could be drawn for each part. In the first part, the water content of the octanol phase decreased rapidly from 4.0% (v/v), which was the saturation value in the phase, with increasing overall glucose concentration up to about 0.2 mol/l. Then, the water content decreased slightly with further addition of glucose in region II. The intersection of the two lines may reflect the change in the property of the separate phase.

We supposed that glucose withdrew water molecules from the octanol phase to form its saturated solution as a separate phase. Using the saturated solubility of glucose in water at 50°C (70.9 g/100 g-solution) [14] and the molar volumes of glucose (0.119 l/mol) [15] and water (0.0182 l/mol), the water concentration in octanol phase $C_{W,org}$ was related to the overall glucose concentration in the system $\bar{C}_G$ by the following equation:

$$C_{W,org} = C_{W,org,O} - n_{W/G}\bar{C}_G \quad (1)$$

where $n_{W/G}$ is the molar ratio of water to glucose in the saturated solution, which was evaluated to be 4.11 from the saturated solubility, $\bar{C}_{W,org,O}$ is the water concentration in the octanol phase at $\bar{C}_G = 0$, and the concentrations in Eq. (1) are expressed in units of mol/l. The water concentration in the octanol phase in units of % (v/v), $\hat{C}_{W,org}$, was related to the $\bar{C}_G$ by using the density of water ρ_W and the molecular weight of water M_W as follows:

$$\hat{C}_{W,org} = \hat{C}_{W,org,O} - \frac{100 n_{W/G} M_W}{\rho_W}\bar{C}_G$$

$$= \hat{C}_{W,org,O} - 7.48\bar{C}_G \quad (2)$$

The $\hat{C}_{W,org,O}$ was determined experimentally to be 4.0% (v/v). The relationship of Eq. (2) is shown by the line *a* in Fig. 1. Eq. (2) expressed the observed $\hat{C}_{W,org}$ when $\bar{C}_G < 0.2$ mol/l. However, the observed $\hat{C}_{W,org}$ values lie over the line in region II. Another explanation, therefore, must be made for this region.

The water activities in the separate and the octanol phases must be the same at equilibrium. The water activity at the saturated glucose concentration $a_{W,sat}$ is constant at a specified temperature. The water activity in the octanol phase must be the same as the $a_{W,sat}$, that is, the water content in this phase is constant when the phase contacts with the saturated glucose solution. We did not measure the water content corresponding to the $a_{W,sat}$, but it was assumed to be 2.2% (v/v) from the intersection of the dotted lines drawn empirically for regions I and II, as shown in Fig. 1. The content is shown by the solid line *b* in Fig. 1. Glucose used was anhydrous. If glucose that was not dissolved was assumed to have a water of crystallization in region II, the water concentration in the octanol phase was given by Eq. (3) from the mass balance in terms of water in the system.

$$C_{W,org} = C_{W,org,O} - n_{W/G}\bar{C}_{G,sat} - n_c\left(\bar{C}_G - \bar{C}_{G,sat}\right) \quad (3)$$

where $\bar{C}_{G,sat}$ is the overall glucose concentration calculated from Eq. (2) at $\hat{C}_{G,org} = 2.2\%$ (v/v). The $\bar{C}_{G,sat}$ was evaluated as 0.24 mol/l. n_c is the number of moles of water of crystallization per glucose molecule, and was set as 1. Thus,

Eq. (3) is rewritten as follows using units of % (v/v):

$$\hat{C}_{W,org} = 2.64 - 1.82\overline{C}_G \quad (4)$$

Equation (4) is shown by line c in Fig. 1. The observed $\hat{C}_{G,org}$ values lay between the lines b and c. However, what actually occurs in region II remains unexplained.

The equality in the water activity between the two phases must also be maintained in region I. This indicates that the separate phase in region I cannot be saturated glucose solution in the strictest sense. However, the assumption of the saturated glucose solution gave a good explanation for the relationship between $\hat{C}_{W,org}$ and $\overline{C}_G$. This may be due to the fact that the water activity of glucose solution decreases steeply near the saturated concentration [16].

As mentioned above, the separate phase could be regarded as a very dense glucose solution in region I and as a saturated glucose solution with undissolved glucose in region II.

The reaction system that included the enzyme may be somewhat different from that shown in this figure because of the presence of the enzyme itself which also has water adsorbability and because of the progress of the reaction. The product may dynamically modify the system. The former might be insignificant when a small amount of the enzyme is used.

3.2. Octyl glucoside synthesis

As shown in our previous paper [11], there would be a possibility that disaccharides were formed in the separate phase. It was, however, impractical to quantify them because of a small volume of the very highly viscous phase. It was shown in the previous paper [11] that the amount of disaccharides formed in a biphasic system decreased as the volume ratio of the organic to aqueous phase increased. Since the present system had a very high ratio although we could not specify the value, we supposed that the formation of disaccharides would not be significant.

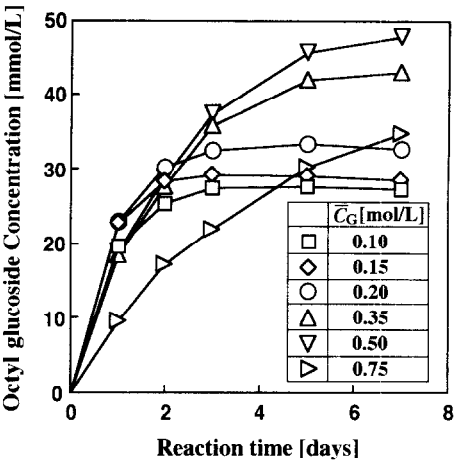

Fig. 2. Effect of glucose concentration on the octyl glucoside synthesis. Reaction was performed in 4 ml of buffer-saturated octanol with 2.5 g/l enzyme at 50°C.

Vulfson et al. [6] reported that disaccharide, cellobiose, gave alkyl β-D-glucoside through transfer reaction and that the approximately same yield of the glucoside was attained at the same concentrations of glucose residue when glucose and cellobiose were used as substrates. The analysis of product was carried out by HPLC using the octanol phase as a sample. It showed only two peaks which corresponded to octanol and reaction product. ^{1}H NMR and FAB-MS analyses of the product recovered by HPLC indicated that the product was octyl-β-D-glucoside. In this context, we focussed on only octyl β-D-glucoside formation in this paper although formation of minor products at the level undetectable by the instrumental analyses could not fully be denied.

Fig. 2 shows the transient changes in octyl glucoside concentration during the synthesis at various initial glucose concentrations. The product concentration increased with increases in glucose concentration. Octyl glucoside formation was low at the highest glucose concentration used (0.75 mol/l). For the first three glucose concentrations, the reaction reached equilibrium within 7 days of incubation, while at the higher concentrations it did not. Since the glucose concentration in the separate phase was

almost at its saturation level at 50°C when the overall glucose concentration was below 0.2 mol/l, the reaction rates were expected to be the same. This can be seen in Fig. 2. The reaction rate seemed to decelerate at glucose concentrations higher than 0.2 mol/l which may reflect a property of the system. The separate phase transformed from the liquid phase to liquid-adsorbed solid-dispersed phase at this concentration. The separate phase became more viscous at higher glucose concentrations, resulting in poor distribution of the separate phase in the bulk octanol phase. Therefore, the reduction of the reaction rate might be attributable to a mass transfer problem.

The octyl glucoside concentration after 7 days of incubation was converted to the yield as shown in Fig. 3. It should be noted again that only the first three points corresponded to the equilibrium yield. The yield decreased with increases in glucose concentration. However, this could be explained by the increase in volume of the separate (aqueous) phase with increases in the amount of glucose added; i.e. by the decrease in volume ratio of the organic to the aqueous phase. As reported in our previous paper [11], this parameter has a significant influence on the equilibrium yield in a two-phase system; the higher the ratio, the higher the yield obtained at equilibrium. The water concentrations of the octanol phase after incubation for 7 days are also shown in Fig. 3.

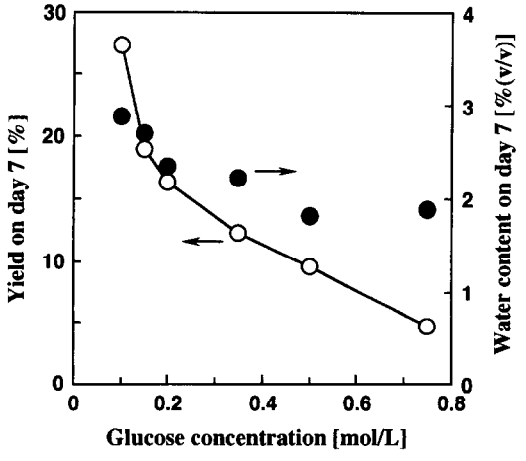

Fig. 3. Yield of *n*-octyl β-D-glucoside and the water content of the octanol phase after 7 days of incubation. The yield was calculated based on the limiting substrate, glucose.

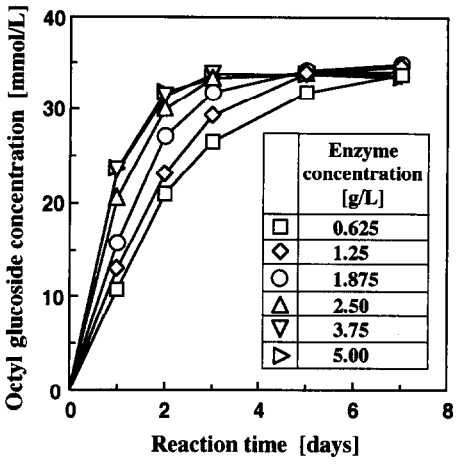

Fig. 4. Effect of β-glucosidase concentration on octyl glucoside synthesis. The condensation reaction was performed in 4 ml of buffer-saturated octanol with an overall glucose concentration of 0.1 mol/l at 50°C

3.3. Effect of enzyme concentration

Octyl glucoside synthesis at various enzyme concentrations is shown in Fig. 4. The results show that the reaction could reach the same equilibrium level independent of the enzyme concentration, although, of course, it affected the reaction rate.

The initial reaction rates of the synthesis were calculated from the data shown in Fig. 4 using numerical differentiation and are shown in Fig. 5. It can be seen that the initial reaction rate was a linear function of the enzyme concentration at low concentrations. When the enzyme concentration was higher than 2.5 g/l, the reaction rate was not proportional to the enzyme concentration and levelled off. When the specific activity was considered, the value decreased linearly at enzyme concentrations greater than 2.5 g/l. Therefore, this enzyme concentration or a lower concentration should be used to optimize the synthesis.

3.4. Reuse of the enzyme

As described above, the enzyme existed in the separate phase containing the very high concentration of glucose. In comparison to our previous study [11] where condensation was carried out with a glucose concentration up to 3.0 mol/l in a biphasic system at 60°C, it was expected that the enzyme in the present system would be more stable due to the stabilizing effect of glucose at the higher concentration [17] and the lower temperature. This was verified by recovering the separate phase containing the enzyme after the reaction reached equilibrium and by reusing this phase for subsequent synthesis. Fig. 6 shows four consecutive syntheses of octyl glucoside at an initial overall glucose concentration of 0.1 mol/l. The initial glucose concentration was adjusted to 0.1 mol/l for subsequent synthesis by addition of glucose equivalent to the product produced in the previous synthesis. The overall yield of the four consecutive runs, based on the total glucose added, reached 58%. The reaction rate of each batch appeared to be the same. However, the equilibrium yield of each batch was decreased slightly. This could be ascribed to an increase in the water content of the system due to the addition of buffer-saturated octanol in each batch. There may be two possible explanations for the gradual decrease in the equilibrium yield in each batch; the glucose concentration in the separate phase may have been reduced by the increase in volume of the phase, or the partition coefficient of the product between the separate

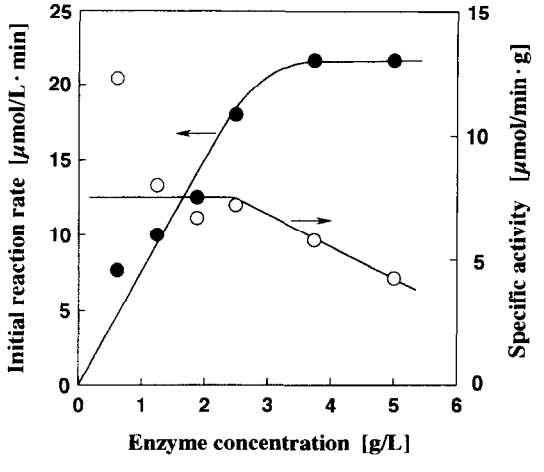

Fig. 5. Catalytic activity of β-glucosidase in the reaction system at 50°C.

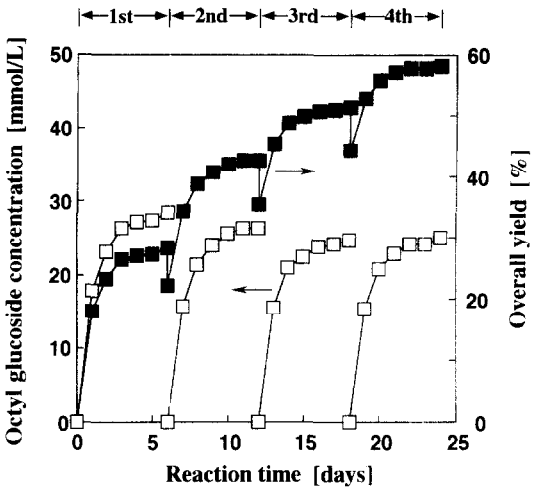

Fig. 6. Repeated use of β-glucosidase for the synthesis of *n*-octyl β-D-glucoside. The enzyme was added at a concentration of 2.5 g/l only in the first batch, and no further addition was made in subsequent batches. The initial overall glucose concentration was adjusted to 0.1 mol/l in each batch.

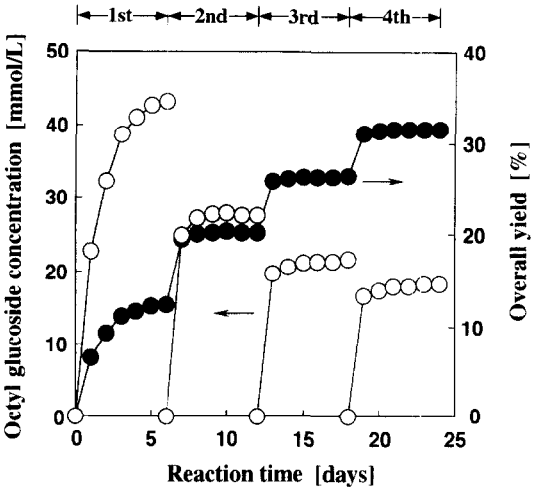

Fig. 7. *n*-Octyl β-D-glucoside synthesis by repeated use of the separate phase. The enzyme and glucose were added at overall concentrations of 2.5 g/l and 0.35 mol/l, respectively, only in the first batch. No further addition of either the enzyme or glucose was made in the subsequent batches.

and the octanol phase may depend on the glucose concentration in the separate phase or on the water content in the octanol phase. Further experiments are required to elucidate which of these is correct.

The enzyme was also reused at the higher initial glucose concentration of 0.35 mol/l for four consecutive syntheses as shown in Fig. 7. After 6 days of incubation in each batch, the separate phase was recovered and reused without any addition of glucose. In this case, the glucose concentration in the system decreased with each batch. The remaining glucose in either liquid or solid form was used as the substrate in the subsequent batch. Although synthesis at a high glucose concentration gave a low yield, the yield could be improved by reusing the separate phase. As shown in this figure, the yield increased from 12% in the first batch to 32% in the fourth batch. It should be noted that the final octyl glucoside concentration in each batch decreased due to a decrease in the amount of glucose and an increase in water content in the reaction system.

4. Conclusion

n-Octyl β-D-glucoside could be synthesized in a system with a very low water content, where buffer-saturated octanol was added to solid glucose and β-glucosidase for the condensation to proceed. In this system, the product was obtained at high concentration and yield. The yield in a batch reaction of this study was higher than or comparable to those reported previously [4–6,8–11]. The presence of glucose in high concentration and the very high volume ratio of the octanol to the separate phase seemed to be the major factors responsible for the increase in yield. The high glucose concentration also resulted in high stability of β-glucosidase even without immobilization. Due to the high viscosity of the separate phase, the phase containing the enzyme and glucose could easily be recovered and reused in subsequent synthesis reactions. The reuse of the aqueous phase containing the enzyme and glucose enabled to achieve much higher yield of the product compared with the previous reports.

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