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Thermal and operational characteristics of glutaryl-7-aminocephalosporanic acid acylase immobilized on silica gel modified by epoxide silanization

Jung Soo Lim[1], Seung Won Park[2] and Seung Wook Kim[1,*]
[1]*Department of Chemical and Biological Engineering, Korea University, 1, Anam-dong, 136-701, Sungbuk-ku, Seoul, Korea*
[2]*Food Ingredient Division, CJ Foods R&D, CJ Corp., 636, Guro-dong, 152-050, Guro-gu, Seoul, Korea*
**Author for correspondence: Tel.: +82-2-3290-3300, Fax: +82-2-926-6102, E-mail: kimsw@korea.ac.kr (Seung-Wook Kim)*

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Summary

In this study, an investigation was performed into the thermal and operational characteristics of glutaryl-7-aminocephalosporanic acid (GL-7-ACA) acylase (EC 3.5.1.-) immobilized on silica gel that had been modified by epoxide silanization. The pH values for the optimum activity of free and immobilized GL-7-ACA acylase were almost the same. However, the pH-dependent activity profile for the immobilized GL-7-ACA acylase is considerably expanded. Both free and immobilized enzymes generally had the highest activity at 50 °C. In thermodynamic studies, it was found that immobilization using epoxide silanization made GL-7-ACA acylase thermodynamically stable. In the results of repeated batch production of 7-ACA, 89.0 and 83.5% of the 7-ACA produced at the initial cycle were maintained after 20 times of recycle at 25 °C and 30 °C, respectively. Hence it was suggested that mass production of 7-ACA at 25 °C using immobilized GL-7-ACA acylase by epoxide silanization would be possible on a large scale.

Introduction

Enzymatic transformation of Cephalosporin C (CPC) into 7-aminocephalosporanic acid (7-ACA) can be performed by a two-step process including the oxidative deamination of CPC to GL-7-ACA (glutaryl-7-aminocephalosporanic acid) catalyzed by a D-amino acid oxidase and the subsequent hydrolysis catalysed by GL-7-ACA acylase (EC 3.5.1.-) (Shin *et al.* 1996; Alfani *et al.* 1997; Bianchi *et al.* 1997; Ezio *et al.* 1998). The 7-ACA produced by GL-7-ACA acylase is a useful intermediate of great commercial interest in the preparation of semi-synthetic cephalosporin antibiotics (Lee *et al.* 1996; Kim *et al.* 2001). The immobilization of the GL-7-ACA acylase can offer several advantages for industrial and biotechnology applications, including the ability to be used repeatedly, the improvement of enzyme stability, and the alteration of the properties of the enzyme (Park *et al.* 2001; Lee *et al.* 2002). In our previous study, the immobilization of GL-7-ACA acylase on silica gel modified by epoxide silanization was investigated (Park *et al.* 2002; Park *et al.* 2003). To develop an efficient process for 7-ACA production, it is essential to characterize the enzyme-stability relationship in great detail.

In recent years, it has been reported that the thermal denaturation of enzymes was accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, with a concomitant increase in the enthalpy of activation, and that the opening up of the enzyme structure was accompanied by an increase in the disorder, randomness or entropy of activation (Daniel 1996; Chen *et al.* 2000; Calsavara *et al.* 2001). These studies showed that immobilization made the enzyme more thermodynamically stable compared to free enzymes when using thermodynamic parameters (Arica & Hasirci 1993; Gawande & Kamat 1998; Rashid & Siddiqui 1998). However, studies have not been reported on GL-7-ACA acylase or the immobilization on silica gel that had modified by epoxide silanization.

In this study, the thermal and operational characteristics of GL-7-ACA acylase immobilized on silica gel modified by epoxide silanization were investigated, and stability was examined by producing 7-ACA with using a repeated batch system.

Materials and methods

Materials

Glutaraldehyde was obtained from Fluka Co. (Switzerland). *p*-Dimethylaminobenzaldehyde was obtained

from KANTO Chemical Co. (Japan). Glutaric anhydride, 3-glycidoxypropyltrimethoxysilane (3-GPTMS) and *N*,*N*-diisopropylethylamine (*N*,*N*-DIPEA) were obtained from Aldrich Co. (USA). 7-ACA was purchased from Sigma Chemical Co. (USA). Ultrafiltration membranes (15659-00-1) were purchased from Sartorius (Germany). GL-7-ACA acylase from genetically engineered *E. coli* BL21 that contains the GL-7-ACA acylase gene of *Pseudomonas* sp. KAC-1 was supplied by Chong Kun Dang Pharmaceutical Corp. (Korea).

Preparation of GL-7-ACA acylase and synthesis of GL-7-ACA

GL-7-ACA acylase and GL-7-ACA were prepared as described in our previous study (Park *et al.* 2003).

Immobilization method of GL-7-ACA acylase

One gram of dry silica gel was mixed in a xylene solution containing 24% (v/v) 3-glycidoxypropyltrimethoxysilane (3-GPTMS) and 3% (v/v) of *N*,*N*-diisopropylethylamine. The suspension was incubated at 80 °C for 2 h with constant mixing, washed thoroughly with de-ionized water before drying at 120 °C for 2 h, after which 10% (v/v) ethylendiamine was added to the silica gel that had been suspended in a 100 mM phosphate buffer solution (pH 8.0) at 90 °C. After stirring (150 rev/min) for 2 h, the suspension was filtered and the carriers were washed with de-ionized water. Glutaraldehyde (1%(v/v)) was added to the silica gel suspended in a 100 mM of phosphate buffer solution (pH 8.0) at 20 °C. After stirring (150 rev/min) for 2 h, the suspension was filtered and carriers were washed with de-ionized water. The activated silica gels were re-suspended in a 100 mM phosphate buffer solution (pH 8.0). Finally, the enzyme solution (10 mg/ml) was added. The suspension was stirred at 20 °C for 2 h and the immobilized GL-7-ACA acylase was recovered by filtration and washed with de-ionized water. After re-suspending the acylase in a 100 mM phosphate buffer solution (pH 8.0), the content of the immobilized enzyme was analyzed by Folin–Lowry method. The amount of protein bound to the carriers was determined by the difference between initial and residual concentrations.

Assay of GL-7-ACA acylase activity

The activity of the immobilized enzyme was measured as described in our previous study (Park *et al.* 2003). One unit of acylase activity was defined as the amount of enzyme that produced 1 μmol of 7-ACA per min at 37 °C, pH 8.0.

Effect of pH and temperature on activity of free and immobilized GL-7-ACA acylase

The effect of temperature on enzyme activity was studied for temperature ranging from 20 to 55 °C with a GL-7-ACA concentration of 25 mM in a phosphate buffer solution (pH 8.0). The effect of pH on the enzyme activity was examined for pH ranging from 5 to 10.

Thermodynamics of the enzyme

Thermal denaturation of the enzyme was determined by incubating the enzyme solutions in 1.0 M phosphate buffer (pH 8.0) at a particular temperature. Aliquots were withdrawn at different times and assayed for enzyme activity as described above. This procedure was repeated at four different temperatures ranging from 37 to 55 °C. The data were fitted to first-order plots and analysed. The first-order rate constants (K_d) were determined by the linear regression of ln (A_i/A_0) vs. t, where A_i is the enzyme activity at time t, A_0 is the initial enzyme activity, and t is incubation time. The thermodynamic data were calculated by using or rearranging the following equations, including the Eyring absolute rate equation (Rashid & Siddiqui 1998):

$$K_d = \left(\frac{K_B T}{h}\right) e^{(-\Delta H^*/RT)} e^{(\Delta S^*/R)} \quad (1)$$

where h (Planck constant) = 6.63×10^{-34} J s and K_B (Boltzman constant, [R/N]) = 1.38×10^{-23} J K^{-1} where N (Avogadros No.) = 6.02×10^{-23} mol^{-1}.

$$\Delta H^* \text{ (enthalpy of activation)} = E_a - RT \quad (2)$$

where$R$ (gas constant) = 8.314 J K^{-1} mol^{-1}.

$$\Delta G^* \text{ (free energy of activation)} = -RT \ln[(K_d \times h)/(K_B \times T)] \quad (3)$$

Equation (3) is derived by rearranging equation (1).

$$\Delta S^* \text{ (entropy of activation)} = (\Delta H^* - \Delta G^*)/T \quad (4)$$

Conversion of GL-7-ACA to 7-ACA

The immobilized enzyme was reacted with GL-7-ACA (25 mM) in a 1.0 M phosphate buffer solution (pH 8.0) at 25 and 30 °C. The progress of the hydrolytic reaction was monitored by HPLC utilizing a 20 mM ammonium acetate solution (pH 5.5) in 2% (v/v) acetonitrile as eluent at a flow rate of 1 ml/min in a μ-Bondapak C18 column (3.9 × 300 mm).

Repeated Batch Production of 7-ACA

The reaction was carried out in a 200 ml Pyrex glass reactor equipped with a sintered glass filter below. Immobilized GL-7-ACA acylase was reacted with 25 mM of GL-7-ACA. Experiments were carried out with working volume of 50 ml containing 5 g of immobilized GL-7-ACA acylase. The procedure was repeated for 20 cycles.

Results and discussion

Effect of pH and Temperature on Immobilized GL-7-ACA acylase

Since enzymes possess many ionizable groups, pH changes will affect the catalytic site and conformation of the enzyme. In general, enzymes are active only over a limited pH range, and in most cases a definite optimum pH is observed. The pH effect on the activity of the free and immobilized forms of GL-7-ACA acylase was investigated within the pH range of 5.0–10.0. The pH values for optimum activity of free and immobilized GL-7-ACA acylase were found to be almost the same (Figure 1). As the pH went either above or below the optimal pH, the activity levels of the free enzyme decreased faster than those of the immobilized GL-7-ACA acylase. When the pH was reduced to pH 6.0, the activity level of the free enzyme lost its activity by about 65%, whereas the immobilized enzyme had only lost about 35% of its activity. When the pH was raised to pH 10.0, the free enzyme had lost approximately 33% of its activity, whereas the immobilized enzyme had only lost approximately 15% of its activity. This implies that the immobilized GL-7-ACA acylase was much more stable to changes in pH than the free GL-7-ACA acylase.

The temperature effect on the free and immobilized GL-7-ACA acylase activity was also studied for temperatures ranging from 20 to 60 °C. Both free and immobilized enzymes had the highest activity at 50 °C (Figure 2). At temperatures above 50 °C, the free and immobilized GL-7-ACA acylase activities showed a sharp decrease, probably due to thermal denaturation of the enzyme. At temperatures both above and below the optimum temperature, the immobilized GL-7-ACA acylase was more resistant to the temperature changes than the free GL-7-ACA acylase. Figure 3 shows thermal stability of the free and immobilized GL-7-ACA acylase at different temperatures. There was no significant decrease of activity of free and immobilized enzymes for 60 min up to 37 °C. At above 37 °C, the stability decreased significantly at both free and immobilized GL-7-ACA acylase. However, the immobilized enzyme was more stable than the free enzyme. When incubated at 50 °C, the immobilized enzyme lost 60% of its activity, whereas the free enzyme lost 77% of its activity. Consequently, the thermal stability of immobilized GL-7-ACA acylase increased significantly compared to that of free GL-7-ACA acylase.

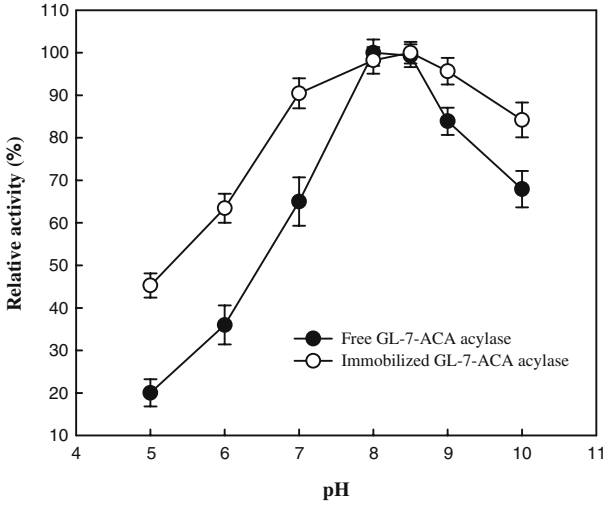

Figure 1. Effect of pH on the activity of free and immobilized GL-7-ACA acylase. Relative activities are expressed as a percentage of the highest activity.

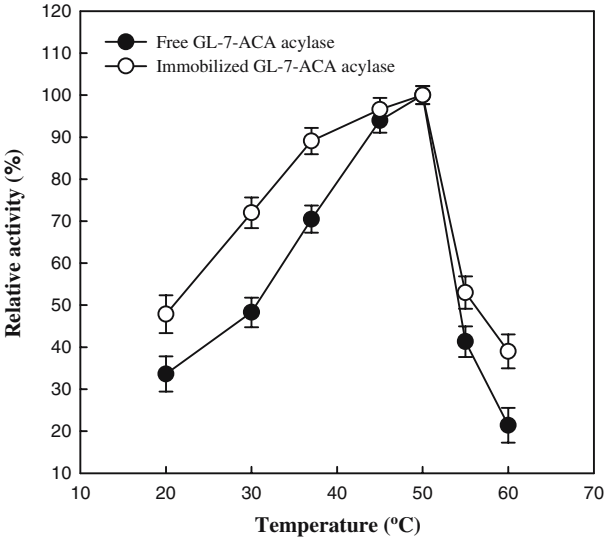

Figure 2. Effect of temperature on the activity of free and immobilized GL-7-ACA acylase. Relative activities are expressed as a percentage of the highest activity.

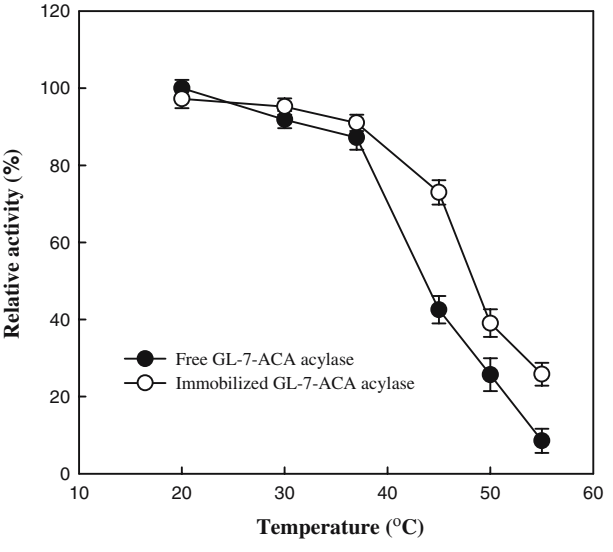

Figure 3. Thermal stability of the free and immobilized GL-7-ACA acylase at different temperatures. Thermal stability was evaluated by measuring the residual activity of GL-7-ACA acylase exposed to each temperature for 60 min. The remaining activities were expressed as a relative percentage of the original activities.

Thermodynamic studies on free and immobilized GL-7-ACA acylase

Tables 1 and 2 show the thermodynamic properties of free and immobilized GL-7-ACA acylase, respectively. Thermal deactivation rate constants (K_d) of the immobilized GL-7-ACA acylase at all temperatures tested in this study were lower than those of the free GL-7-ACA acylase, and half-life times ($t_{1/2}$) were higher than those of the free enzyme.

In recent years, the thermostability of enzymes has been investigated by thermodynamic parameters such as enthalpy, entropy, and free energy of activation (Rashid & Siddiqui 1998; Chen *et al.* 2000; Saleem *et al.* 2005). Thermodynamic study is essential to describe enzyme–stability relationship in detail. So, the thermodynamic parameters of free and immobilized GL-7-ACA acylase were compared at different temperatures. The immobilized GL-7-ACA acylase showed lower enthalpy and entropy of deactivation than those for the free GL-7-ACA acylase at all temperatures tested. This result indicates that immobilization caused a decrease in the values of both thermodynamic parameters. Increased free energy of deactivation also indicates that the immobilization made the enzyme more stable thermodynamically than the free enzyme. It has been reported that decreased enthalpy and entropy with immobilization of the enzyme was related to the changes in conformation, unfolded transition states, and the hydrophobic core of the enzyme with a corresponding increase in thermostability (Daniel 1996; Rashid & Siddiqui 1998). These results suggest that immobilized GL-7-ACA acylase has higher thermal stability than the free enzyme, and that GL-7-ACA acylase immobilized on silica gel modified by epoxide silanization can successfully be used for the production of 7-ACA on an industrial scale at high temperatures.

Conversion of GL-7-ACA into 7-ACA and repeated batch production of 7-ACA using immobilized GL-7-ACA acylase

To observe operational characteristics, the conversion rate of GL-7-ACA into 7-ACA by GL-7-ACA acylase immobilized on silica gel modified by epoxide silanization was investigated at 30 °C and 25 °C. A 25 mM of GL-7-ACA was poured into a 1.0 M phosphate buffer solution (pH 8.3) containing the immobilized GL-7-ACA acylase (21.56 U/g-matrix). Figure 4 (a) shows the conversion of GL-7-ACA acylase immobilized on silica gel into 7-ACA at 30 °C. About 95% conversion of GL-7-ACA was obtained after 30 min and 21.39 mM of 7-ACA was produced at 30 °C. As shown in Figure 4 (b), about 93% conversion of GL-7-ACA was obtained after 50 min and 20.39 mM of 7-ACA was produced at 25 °C.

On the basis of the above results, repeated batch production of 7-ACA was investigated at 25 and 30 °C for 50 min. As shown in Figure 5, twenty cycles were carried out in succession, operating in a discontinuous batch mode. The 7-ACA produced in the first cycle was 20.06 mM, and 89.0% of the initial cycle was maintained after being recycled 20 times at 25 °C. On the other hand, at 30 °C, the 7-ACA produced in the first cycle was 21.90 mM, and 83.5% of the initial cycle was maintained after being recycled 20 times. Through the above results, it seems that the production of 7-ACA at 25 °C was more efficient in terms of the long-term stability than that at 30 °C, although the initial production of 7-ACA at 30 °C was higher than that at 25 °C. These

Table 1. Thermodynamic and kinetic parameters of free GL-7-ACA acylase.

T (K)	$K_d \times 10^2$ (min^{-1})	$t_{1/2}$ (min)	ΔG^* (kJ mol^{-1})	ΔH^* (kJ mol^{-1})	ΔS^*(J mol^{-1} K^{-1})
310	0.22 ± 0.04	307.65 ± 9.5	91.79	27.55	–207.23
318	1.38 ± 0.02	49.97 ± 8.4	100.2	27.48	–228.68
323	2.33 ± 0.02	29.72 ± 7.3	100.4	27.43	–225.91
328	4.07 ± 0.03	17.03 ± 5.1	100.5	27.39	–222.90

K_d is the first-order rate constant for deactivation; ± indicates the standard deviation of three independent readings. Their values under column K_d and $t_{1/2}$ were too small and have not been presented. ΔH^* (kJ mol^{-1}) = E_a (30.12 kJ mol^{-1}) – RT. E_a (activation energy of denaturation) is calculated from the Arrhenius plot of denaturation of free GL-7-ACA acylase. ΔG^* (kJ mol^{-1}) = $-RT \ln[(K_d \times h)/(K_B \times T)]$. ΔS^*(J mol^{-1} K^{-1}) = $(\Delta H^* - \Delta G^*)/T$.

Table 2. Thermodynamic and kinetic parameters of immobilized GL-7-ACA acylase.

T (K)	$K_d \times 10^2$ (min^{-1})	$t_{1/2}$ (min)	ΔG^* (kJ mol^{-1})	ΔH^* (kJ mol^{-1})	ΔS^*(J mol^{-1} K^{-1})
310	0.17 ± 0.03	407.73 ± 10.1	103.01	16.67	–278.52
318	0.54 ± 0.04	128.86 ± 9.8	102.68	16.60	–270.69
323	1.41 ± 0.01	49.51 ± 7.1	101.76	16.56	–263.78
328	2.02 ± 0.03	34.66 ± 6.2	102.40	16.52	–261.83

K_d is the first-order rate constant for deactivation; ± indicates the standard deviation of three independent readings. Their values under column K_d and $t_{1/2}$ were too small and have not been presented. ΔH^* (kJ mol^{-1}) = E_a (19.25 kJ mol^{-1}) – RT. E_a (activation energy of denaturation) is calculated from the Arrhenius plot of denaturation of immobilized GL-7-ACA acylase.

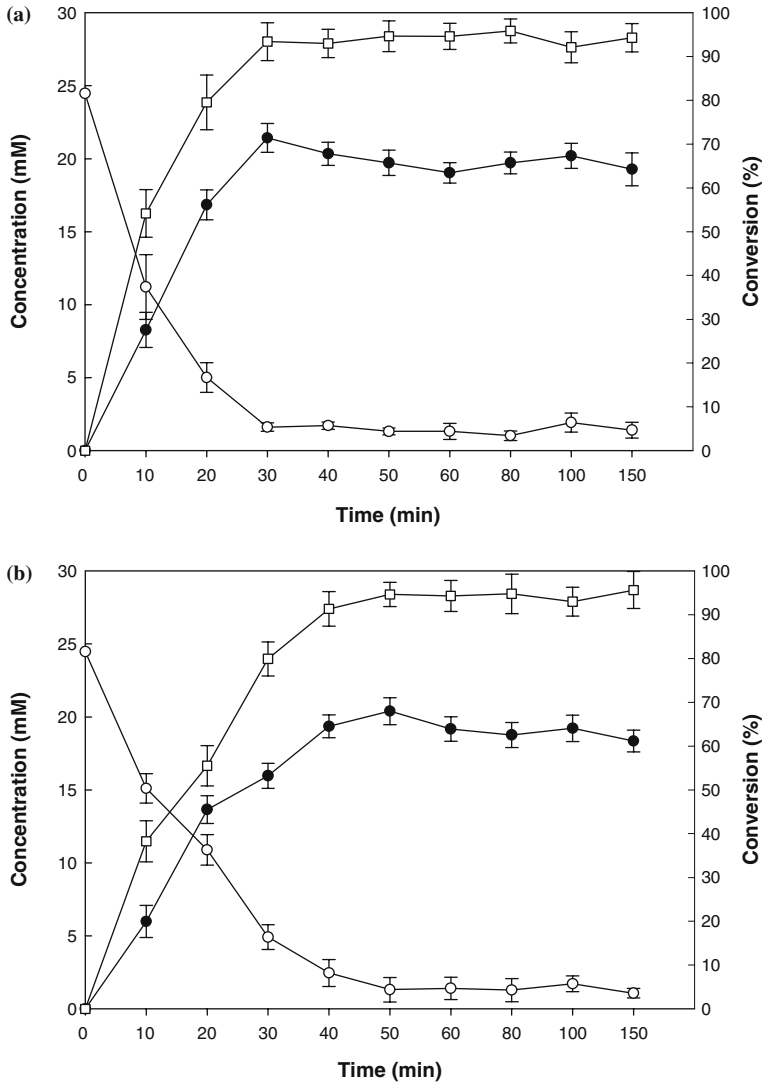

Figure 4. Enzymatic transformation of GL-7-ACA at (a) 30 °C and (b) 25 °C. 7-ACA (●) was produced from GL-7-ACA (○) using GL-7-ACA acylase immobilized on 5 g of silica gel modified by epoxide silanization was reacted with 25 mM of substrate. Conversion (□) = Concentration of reacted substrate/Concentration of initial substrate×100.

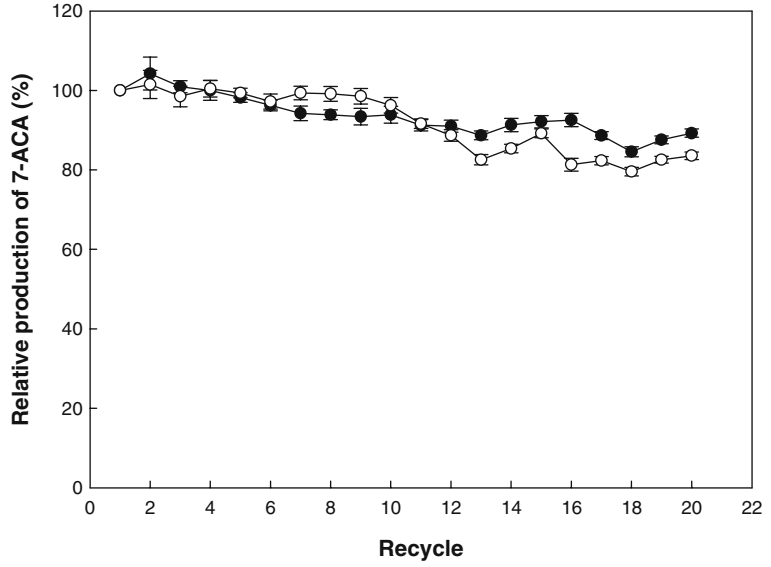

Figure 5. Repeated batch production of 7-ACA in a 200 ml glass reactor. Experiments were carried out with a working volume of 50 ml containing 5 g of immobilized GL-7-ACA acylase. Each cycle at 25 °C (●) and 30 °C (○) was conducted for 50 min.

results are important for industrial application because the optimal operation temperature has a significant effect on 7-ACA production, and the immobilization used in this study should be applicable to the mass production of 7-ACA in large scales.

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