J. Microbiol. Biotechnol. (1998), 8(2), 165 – 170

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY

On-off Dewatering Control for Lipase-catalyzed Synthesis of *n*-Butyl Oleate in *n*-Hexane by Tubular Type Pervaporation System

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Received: December 3, 1997

Abstract Lipase-catalyzed esterification of *n*-butyl oleate was carried out in *n*-hexane as a model reaction. The optimal activity of *Candida rugosa* lipase was shown in a water activity (a_w) range of 0.52 to 0.65 at 30°C. The water produced from the esterification was removed by a tubular type pervaporation system. The rate of ester formed from the enzymatic esterification was allowed to be the same as the rate of water removal by maintaining an optimal a_w of the reaction system using an on-off dewatering control device. The reaction rate and yield with a_w control were increased two folds higher than the respective values for the uncontrolled reaction.

Key words: Lipase-catalyzed esterification, *n*-butyl oleate, tubular type pervaporation system, on-off dewatering control

Enzymatic biotransformations have been widely studied because enzymatic processes have the following advantages: (i) conservation of energy; (ii) minimization of thermal degradation; (iii) regioselectivity and enantioselectivity of enzymes; and (iv) non-toxicity of enzymes [9]. The thermodynamic equilibrium of hydrolytic enzymes can also be shifted towards the synthesis reaction by carrying out the reaction in a non-aqueous system [7]. Therefore, enzymatic syntheses in non-aqueous systems have been widely applied in the biotransformation of fats and oils, synthesis of structured triglycerides and emulsifiers, peptide synthesis, oligosaccharide synthesis, sugar modification [13], and optically pure drug syntheses [5]. In these reactions, water plays a very important role in the activity of the enzymes and the stability of this activity. Especially, in enzymatic esterification, water is continuously produced, resulting in an increase in the water activity (a_w) of the reaction mixture and a shift of the reaction equilibrium in favor of hydrolysis. The increase in a_w can also decrease the rate of synthesis [1, 12]. In order to maintain the equilibrium in favor of synthesis and to run the reaction efficiently, it is necessary to remove the water and control the a_w. Salt hydrates have been successfully used for the removal of water and the control of a_w during synthesis [3, 14]. However, these methods were reported to have some problems concerning practical utility due to the possibility of poisoning of the enzyme by some of the salt used, the reactivity of the salt with the reaction substrates, salt toxicity, and a difficult recovery and reuse of the salt and/or the enzyme in the economical operation of a large-scale reactor [10]. Recently, pervaporation was used to selectively remove water formed from lipase-catalyzed esterifications in organic solvents [8], but a_w control was difficult since no feasible a_w sensor was available and the a_w sensor had limitations in stability, sensitivity, and measurement range in organic solvents [6]. In this report, we described a computer-controlled pervaporation system for controlling the a_w. A tubular type membrane system was used instead of the sheet type membrane system [2, 8]. Compared with the sheet type system previously used [8], the tubular type system has several advantages in its durability at relatively high temperature and high flow rate, and in its ability to withstand exposure to harsh chemical [11]. Furthermore, the membrane area exposed to the reaction mixture can be increased in this tubular type pervaporation system because several tubular type membrane modules are inserted into the enzyme reactor. However, the membrane area cannot be increased in the sheet type system because the area is localized on the bottom of the reactor. The on-off control of the pervaporation system was used for a continuous a_w

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control. This control made it possible to perform the lipase-catalyzed esterification without changing the a_w of the reaction mixture, and the reaction was able to proceed towards synthesis in the irreversible reaction mode.

MATERIALS AND METHODS

Materials

Lipase from *Candida rugosa* (80 μmol fatty acids liberated $min^{-1}mg^{-1}$ by the hydrolysis of olive oil at pH 7.0 at 30°C) was kindly donated by Meito Sangyo. Oleic acid was obtained from Junsei Chemical Co. (Tokyo, Japan). Butanol and cellulose acetate were purchased from Aldrich Chemical Co. (Milwaukee, U.S.A.). *n*-Hexane from Junsei Chemical Co. (Tokyo, Japan) was used as the organic medium. Salt hydrates used were $Na_2HPO_4.2/0$ (a_w=0.18), NaAc.3/0 (a_w=0.3), $Na_4P_2O_7.10/0$ (a_w=0.52), $Na_2HPO_4.7/2$ (a_w=0.65), $Na_2HPO_4.12/7$ (a_w=0.85). The a_w in parentheses is the value at 30°C [3].

Tubular Type Membrane Module Preparation

Tubular type porous ceramic support (average pore diameter was about 0.1 μm) was prepared. The porous support was coated with 20 wt% of cellulose acetate solution in acetone using the dip-coating and rotation-drying technique (Fig. 1). The thickness of the active layer of the cellulose acetate was about 30 μm [11].

Determination of Optimal Water Activity

In order to determine the optimal a_w, 50 mg lipase and 400 mM substrate solution (1:1 molar ratio of oleic acid to butanol in *n*-hexane) were separately pre-equilibrated with the same salt hydrates by direct contact for three days in a cylindrical reactor with screw caps and teflon-lined septa. The reaction was initiated by adding 25 ml of the pre-equilibrated substrate solution into the cylindrical reactor in which 50 mg of lipase and 2 g of the salt hydrates were included, and then mixed vigorously on a shaker (175 strokes/min) at 30°C. The reaction progress was monitored by GC.

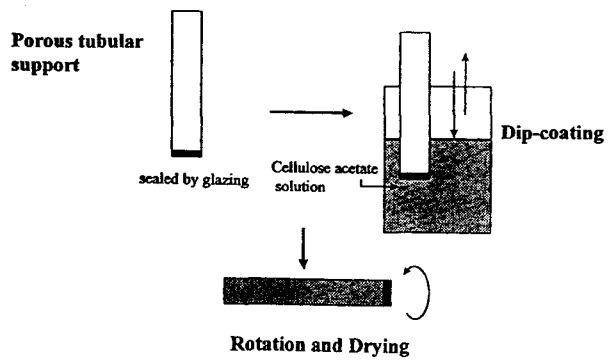

Fig. 1. Preparation of the tubular type membrane.

Determination of Sorption Isotherm Curve of the Reaction Mixture

The reaction mixture consisted of 400 mM oleic acid, 400 mM butanol, and 25 ml *n*-hexane. The reaction mixtures were pre-equilibrated with 2 g of different salt hydrates at 30°C. The water contents of the pre-equilibrated substrate solutions were analyzed on a Karl Fisher titrater. The sample volume for the Karl Fisher titration was 100 μl.

Determination of Dewatering Rate of Reaction Mixture by Pervaporation

The reaction mixture was pre-equilibrated with water (a_w=1). 25 ml of the water saturated reaction mixture was added to the pervaporation system. The water in the reaction mixture was removed by pervaporation at 30°C. 500 μl samples were taken at the pre-determined time intervals. The water contents of the samples were analyzed on the Karl Fisher titrater.

Gas Chromatography Analysis

Samples taken from the reaction were analyzed by a Hewlett-Packard Model 5890 series II GC with a flame ionization detector. The nonpolar column used was a Quadrex 65HT capillary column (25 m × 0.25 mm i.d., film thickness: 0.1 μm) coated with 65% phenyl and 35% methyl silicone gum. Helium was used as a carrier gas. The column flow rate was 1 ml/min, and split ratio was 125:1. 1 μl of sample was injected. Injector and detector temperature were 300°C, and the following temperature program was used: 180°C to 190°C (0.5°C/min).

Lipase-catalyzed Esterification with Continuous a_w Control

Lipase (50 mg) and substrate solution (400 mM) were pre-equilibrated separately with $Na_2HPO_4.7/2$ (a_w=0.65) in the vapor phase and with direct contact, respectively, for three days before mixing. The reaction was initiated by adding 25 ml of the pre-equilibrated substrate solution to the cylindrical reactor in which the pre-equilibrated lipase was included, and mixed vigorously on a shaker (175 strokes/min) at 30°C for 480 min. During the reaction, no salt hydrates were included in the reactor. Samples were taken at predetermined time intervals. The reaction progress was monitored by GC, and the water content was analyzed on the Karl Fisher titrater. A tubular type pervaporation system was inserted into the enzyme reactor to remove the water produced from the lipase-catalyzed esterification (Fig. 2). On-off dewatering control was carried out to remove the water without changing the a_w in the reaction mixture.

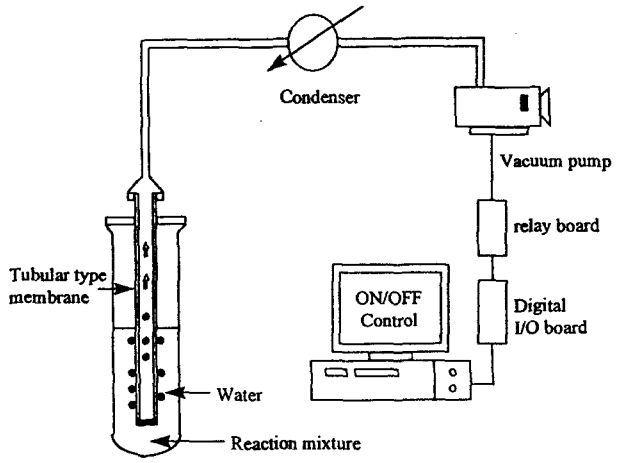

Fig. 2. Schematic diagram of the enzyme reactor system for the on-off dewatering control. Abbreviation: I/O, Input/Output.

RESULTS AND DISCUSSION

Effect of Water Activity on the Lipase-catalyzed Esterification

It is well known that a_w is a critical parameter in lipase-catalyzed esterification since the synthetic activity is strongly dependent on it [4]. In order to determine the optimal a_w of *C. rugosa* lipase, the lipase-catalyzed esterification of *n*-butyl oleate was carried out at 30°C. Because salt hydrates can maintain a constant a_w [3], various salt hydrates were added directly to the reaction mixture. The optimal water activity was found to be in the range of 0.52 to 0.65 (Fig. 3), as the lipase activity was optimal at this range. The water solubility range

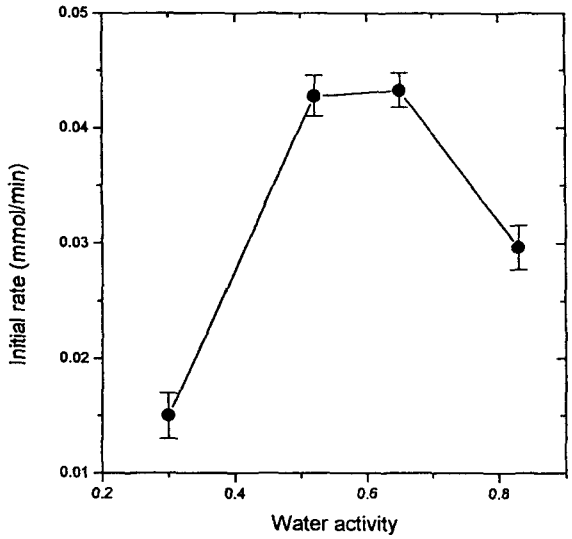

Fig. 3. Effect of water activity on the lipase-catalyzed esterification of n-butyl oleate.
Reaction mixtures contained 400 mM butanol, 400 mM oleate, 2 g different salt hydrates, and 50 mg *C. rugusa* lipase in 25 ml *n*-hexane.

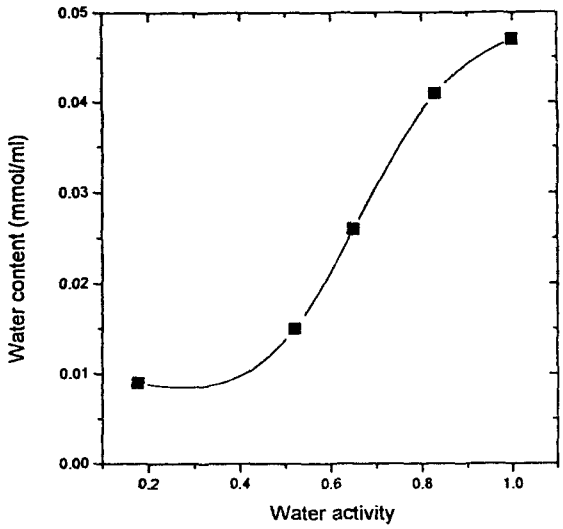

Fig. 4. Sorption isotherm curve for the reaction mixture.
Reaction mixtures contained 400 mM butanol, 400 mM oleate, and 2 g different salt hydrates in 25 ml *n*-hexane.

corresponding to the optimal a_w range was 0.015 to 0.026 mmol/ml (Fig. 4).

The Dewatering Rate of the Reaction Mixture By Pervaporation

Pervaporation was carried out to remove the water in the reaction mixture. The tubular type membrane module, rather than the sheet type membrane module previously developed [8], was used. The regulation of water removal was essential for the control of the overall a_w during the lipase catalyzed esterification since water was continuously formed as the esterification proceeded. Investigation of the dewatering rate by pervaporation was needed for the control of water removal. As shown in Fig. 5a, the dewatering rate was high at high water content, but low at low water content. The semi-log plot of water concentration vs. time (Fig. 5b) can be expressed by the following linear equation:

$$\log[\text{water(mmol/ml)}] = -1.367 - 0.037 \times \text{Time (min)} \quad (1)$$

Strategies for Controlling a_w in Lipase-catalyzed Esterification

As mentioned above, the lipase-catalyzed esterification was greatly affected by a_w. Furthermore, it was found that the a_w of the reaction mixture is very important for high reaction rate and yield. Therefore, it was important to maintain the desired a_w during the reaction. However, the a_w in lipase-catalyzed esterifications continuously changes because water is continuously formed as the reaction proceeds as follows:

$$R_1COOH + R_2OH \longrightarrow R_1COOR_2 + H_2O \quad (2)$$

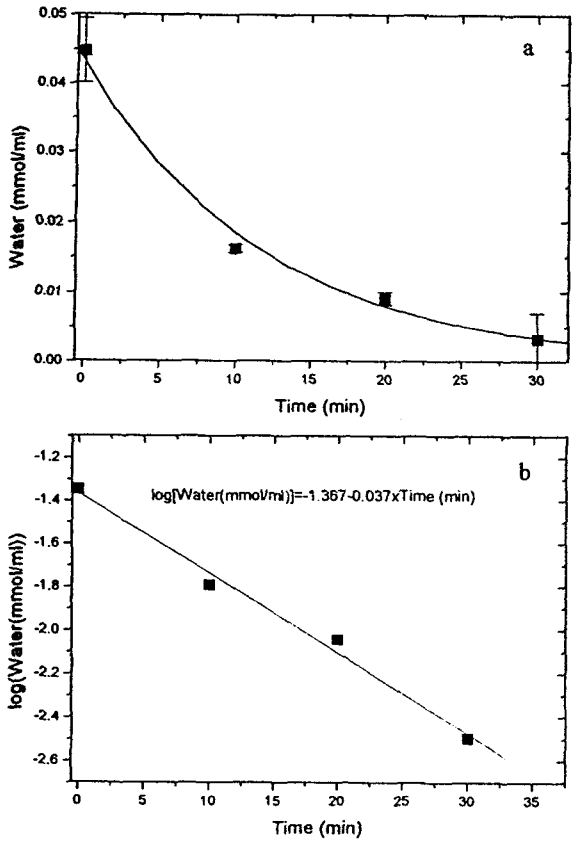

Fig. 5. Dewatering rate of the reaction mixture by pervaporation.
Linear-linear (a) and linear-log (b) plot. The reaction mixture contained 400 mM butanol and 400 mM oleate in 25 ml *n*-hexane.

where R_1 and R_2 are acyl moieties. In order to overcome the problem, the removal of water was performed by pervaporation with an on-off control for maintaining the optimal a_w. The dewatering control algorithm was as follows:

1. If water was removed at the same rate as ester formmation using pervaporation with optimal a_w maintenance, the original reversible reaction would be performed in the irreversible reaction mode as follows:

$$R_1COOH + R_2OH \xrightarrow{k} R_1COOR_2 \quad (3)$$

The reaction rate is shown below:

$$\frac{d[Ester]}{dt} = k[FA][OH] \quad (4)$$

where [FA] is R_1COOH concentration (mmol/ml), [OH] is R_2OH concentration (mmol/ml), and [Ester] is R_1COOR_2 concentration (mmol/ml).

2. The reaction rate constants (k) at different water activities were obtained from the experiment that controlled a_w using salt hydrates (Table 1). The k value at the optimal a_w was 0.01075 ± 0.00038 ml/ mmol·min at 30°C.

Table 1. Reaction rate constant (k) of *C. rugosa* lipase-catalyzed esterification of *n*-butyl oleate in *n*-hexane at different water activities.

a_w buffer used	a_w	rate constant (k) (ml/mmol·min)
NaAc.3/0	0.3	0.00375 ± 0.00050
$Na_4P_2O_7$.10/0	0.52	0.01070 ± 0.00045
Na_2HPO_4.7/2	0.65	0.01075 ± 0.00038
Na_2HPO_4.12/7	0.8	0.00742 ± 0.00048

3. The ester concentration was integrated by the fourth order adaptive step size Runge-Kutta method (Eq. 5). The ester concentration produced in 1 min, $F(t_n, t_{n+1})$, is shown in Eq. (6);

$$[Ester] = \int_0^t k[FA][OH]\,dt \quad (5)$$

$$F(t_n, t_{n+1}) = \int_{t_n}^{t_{n+1}} k[FA][OH]\,dt \quad (6)$$

where $t_{n+1}-t_n=1$ min.

4. Water was removed so as to maintain the optimal a_w range of 0.52 to 0.65 in which lipase activities were optimal (Fig. 3). On-off dewatering control was performed to maintain the optimal a_w range (0.52 to 0.65). The water solubility (S) rather than a_w was controlled by the on-off dewatering control. The water solubility (S) was kept in the optimal a_w range. From Eq. (1), the water content removed by dewatering for 1 min, ΔS, is shown in the following Eq. (7):

$$\Delta S = S \times (1 - 10^{-0.037 \times (t_{n+1} - t_n)}) \text{ (mmol/ml)} \quad (7)$$

where $t_{n+1}-t_n=1$ min.

When both water formed from the enzymatic esterification and water removed from the dewatering by pervaporation are considered, the water solubility (S) can be expressed as the following Eq. (8). When water was not removed, ΔS was zero.

$$S + F(t_n, t_{n+1}) - \Delta S = S \quad (8)$$

where $F(t_n, t_{n+1})$ is the water concentration actually produced from the esterification in 1 min because water is formed at the same rate as ester formation. $F(t_n, t_{n+1})$ was calculated from Eq. (6).

Figure 6 shows the on-off dewatering control algorithm. The initial water solubility (S_0) was 0.026 mmol/ml. The solubilities of the upper criteria (S_U) and lower criteria (S_L) were fixed at 0.026 and 0.015 mmol/ml, respectively, because the water solubility range corresponding to the optimal a_w range was 0.015 to 0.026 mmol/ml. If the water solubility increased above S_U, a switch was turned on, while if the water solubility decreased below S_L, the switch was turned off. The time course for the water solubility calculated from the algorithm is shown in Fig. 9. The on-off state of the pervaporation system is shown in Fig. 7.

The Lipase-catalyzed Esterification of *n*-Butyl Oleate with a_w Control

The lipase-catalyzed esterification of *n*-butyl oleate was carried out in the reaction system with the on-off dewatering control to prove the validity of the developed on-off algorithm (Fig 6). The rate of the controlled reaction was about twice as fast as that of the uncontrolled reaction, and the experimental data fit the ester curve integrated from the Eq. (5) quite well (Fig. 8). In addition, the water solubility curve calculated from the dewatering control algorithm fit the experimental data well (Fig. 9). Therefore, the water formed from the enzymatic esterification could be removed so as to maintain the optimal a_w range of the reaction mixture, and the reaction was able to be proceeded toward synthesis in the

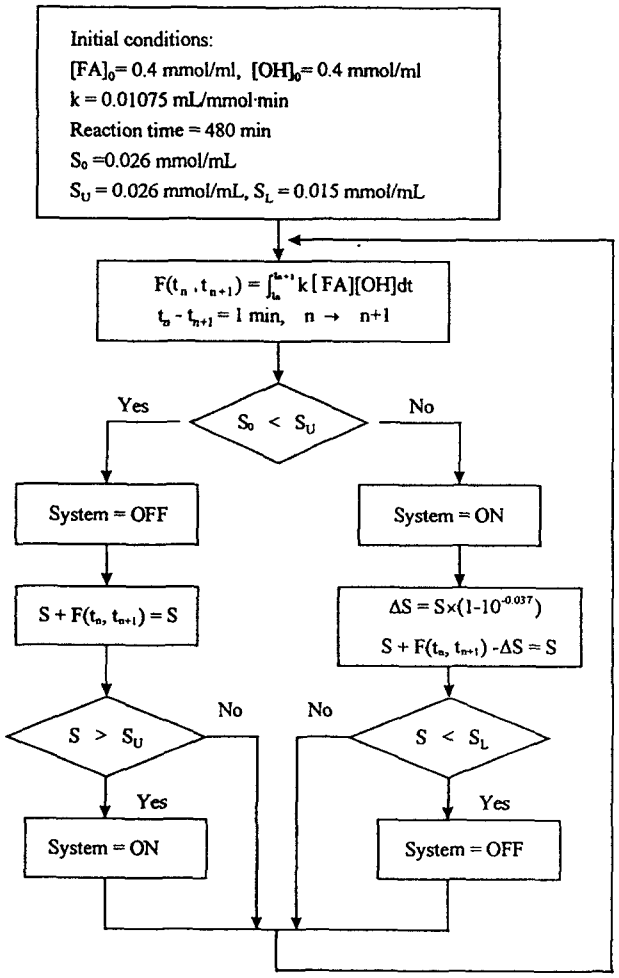

Fig. 6. The on-off dewatering control algorithm.

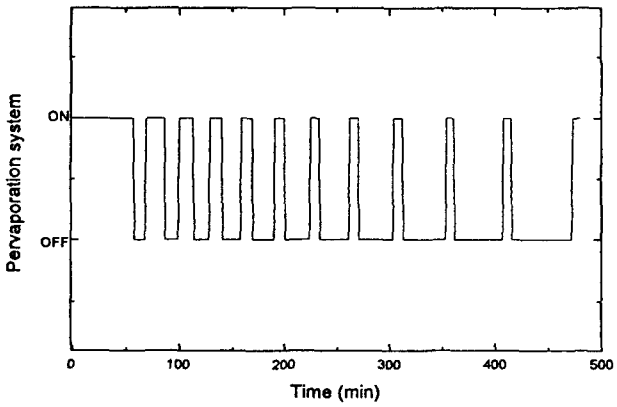

Fig. 7. Time course for the on-off state of the pervaporation system designed from the algorithm shown in Fig. 6.

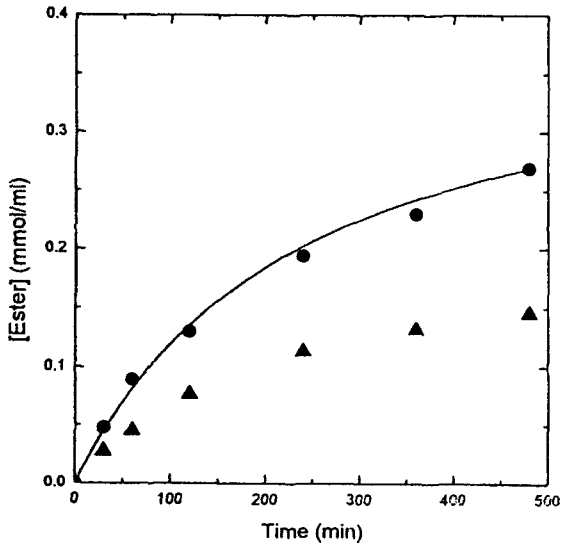

Fig. 8. Time course for the lipase-catalyzed esterification of *n*-butyl oleate with the on-off dewatering control (●) or without control (▲).

Solid line: ester concentrations integrated from the adaptive step size Runge-Kutta method (equation 5). Reaction mixtures contained 50 mg *C. rugusa* lipase, 400 mM butanol, and 400 mM oleate in 25 mL *n*-hexane.

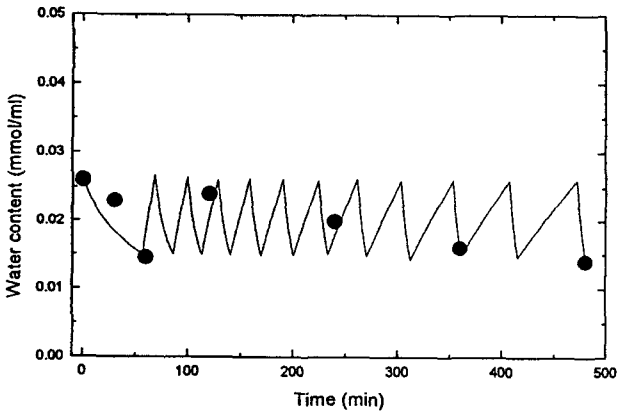

Fig. 9. Time course for the water content in the reaction mixture.

The water contents of the reaction mixtures (●) were analyzed on the Karl-Fisher titrater. Reaction conditions are described in Fig. 8. The solid line was the water solubility curve of the reaction mixture calculated from the algorithm shown in Fig. 6.

irreversible reaction mode. We hope that this method can be used to control the a_w in various enzymatic syntheses of esters, peptides, and glycosides in the presence of organic solvents where direct a_w control is impossible.

Acknowledgments

We are grateful to Mr. S. H. Kim for his help with the computer work of this study. One of the authors (S. J. Kwon) also appreciates the financial support from American Oil Chemists' Society (Unichema Scholarship).

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