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Kinetic investigation on asymmetric bioreduction of ethyl 4-chloro acetoacetate catalyzed by baker's yeast in an organic solvent-water biphasic system

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Abstract−This study investigated the kinetic characteristics of asymmetric bioreduction of ethyl 4-chloro acetoacetate (ECA) to produce (S)-4-chloro-3-hydroxybutyric acid ethyl ester (S-CHBE) by baker's yeast in a water-organic solvent biphasic system. Exactly how several organic solvents affect reaction performance was studied first. Among the solvents tested, petroleum ether exhibited the optimum reaction efficiency. Compared with the aqueous system, reaction yield was enhanced from 74.5% to 84.0%, and the product's ee increased from 82.3% to 88.0% after 10% petroleum ether was added. The kinetic behavior of asymmetric bioreduction of ECA in the petroleum ether-water biphasic system was then examined by using a mathematical model. Kinetic analysis reveals that the maximal reaction rate and affinity between the substrate and the biocatalyst were both lower in the biphasic system than in the aqueous system. Additionally, the substrate inhibition effect was greater in this biphasic system than in the aqueous system. However, the ratio of the formation rate for producing S-CHBE to that for producing R-CHBE in the biphasic system was significantly higher than that in the aqueous system. Moreover, adding petroleum ether reduced spontaneous ECA degradation markedly. These two kinetic characteristics explain why the biphasic system exhibited a higher yield and a better product's ee (enantiomeric excess) than the aqueous system.

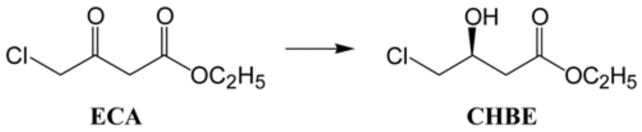
Key words: Asymmetric Reduction, Baker's Yeast, Biphasic System, Substrate Stability, Substrate Inhibition

INTRODUCTION

The asymmetric reduction of ketones by baker's yeast has been studied extensively to produce chiral alcohols because the biocatalyst is inexpensive, readily available and non-toxic, as well as its wide substrate specificity and, more importantly, the possibility of cofactor regeneration, which is essential in sustaining catalytic activity in the cells [1-3]. However, since enzymes inside yeast cells include different or opposite stereospecificities that may reduce the keto substrates to different enantiomeric products, baker's yeast does not frequently produce the desired configuration of β-hydroxy ester with high optical purity and in a high reaction yield. Furthermore, substrate/product auto-degradation and substrate inhibition effect on the enzymatic activity are often observed in these bioreduction systems [4-6].

S-4-Chloro-3-hydroxybutyric acid ethyl ester (S-CHBE) is an important optically active synthon in the pharmaceutical industry [7,8]. This chiral synthon can be produced by the asymmetric reduction of ethyl 4-chloroacetoacetate (ECA) with baker's yeast (Scheme 1). In this bioreduction system, a severe substrate auto-degradation [9,10] and a mild substrate inhibition effect [4] were experimentally observed.

Alternatively, adding organic solvent to an aqueous medium may be a feasible means of weakening the substrate inhibition effect and reducing auto-degradation of the substrate. Moreover, adding an organic solvent often increases substrate solubility and, most importantly, alters the stereospecificity of baker's yeast [11-14]. To date, however, the kinetic behavior of such a biphasic system has seldom been characterized.



Scheme 1.

Our previous work successfully developed a mathematical model that simulates the asymmetric reduction of ECA catalyzed by baker's yeast in an aqueous medium [15]. The model considers the kinetics of enzymatic reaction, the effect of substrate inhibition, and the spontaneous degradation of the substrate. In this work, the enantioselective bioreduction of ECA in a water-organic solvent biphasic system is elucidated. The kinetic characteristics of this biphasic system are investigated by using the mathematical model.

EXPERIMENTAL

1. Cultivation of Yeast Cells

Saccharomyces cerevisiae was isolated from the fresh pressed yeast product of Yung Chang Co. (Taipei, Taiwan). The yeast cells were pre-cultured to the late log phase at 30 °C on an orbital-shaker at 200 rpm in a modified YM medium containing 9 g/L yeast extract, 9 g/L malt extract, 15 g/L peptone, 20 g/L glucose (pH 7.0). Then 25 mL of pre-cultured broth was transferred into a 2-liter Erlenmeyer flask containing 225 mL of modified YM medium. Flask culture was carried out at 30 °C, 200 rpm to the late exponential

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phase (about 15 h). Finally, the cultivated cells were collected by centrifugation at 6,000×g for 25 min.

2. Yeast-Catalyzed Reduction Reaction

The reduction reaction was carried out in an organic solvent-Tris/HCl buffer (0.3 M, pH 8.5) biphasic solution in a total volume of 20 mL. The reaction solution contained yeast cells, glucose and a certain amount of ethyl 4-chloro acetoacetate (Lancaster Synthesis Co., Lancashire, UK). The reduction reaction proceeded in a shaking water bath at 190 rpm. At each time interval, one flask was taken and the reaction was quenched by adding 2 mL of 6 N H_2SO_4 solution. Next, isopropanol was added to make the biphasic solution into a homogeneous phase mixture. The yeast cells were separated by centrifugation (8,000×g for 10 min). A certain amount of the supernatant was diluted with isopropanol before the analysis of the substrate and product concentrations by gas chromatography (GC). After isopropanol was removed under reduced pressure, the pH of the residual supernatant was adjusted to 6.5, followed by extraction twice with equal volumes of ethyl acetate (EA). The organic portion was separated and dried over anhydrous magnesium sulfate powder. The EA extract was then concentrated under reduced pressure at 40 °C and the oily residue was used for optical purity determination. Concentrations of the substrate and product and the product's optical purity were analyzed by the methods described previously [16].

The reaction yield was estimated from the ratio of the product formation (including S- and R-CHBE) to its initial substrate concentration. The optical purity was expressed by the enantiomeric excess (ee) value. The initial reaction rate was estimated as the product formation rate at the beginning of the reaction. Each experiment was performed in triplicate.

RESULTS AND DISCUSSION

1. Selection of Organic Solvent

Table 1. Effect of organic solvents on the reaction performance of ECA bioreduction in water-organic solvent biphasic system[a]

Solvent	Log P (-)	Yield (%)	ee (%)
Control[b]	-	74.5	82.3
Methanol	−0.76	38.3	78.1
Acetonitrile	−0.33	31.2	81.2
Ethanol	−0.24	27.6	84.7
n-Butanol	0.80	12.3	89.1
Hexanol	1.86	6.9	83.0
Toluene	2.60	24.6	84.7
Cyclohexane	3.20	78.0	80.9
Petroleum ether	3.50	80.8	83.5
n-Hexane	3.52	67.1	82.8
n-Octane	4.50	21.6	83.4

[a]Reaction conditions: 6.0 g wet yeast cells, 0.54 g glucose, 18.0 mL Tris/HCl buffer (0.3 M, pH 8.5), 2.0 mL organic solvent containing ECA (the final concentration was 73.5 mM), 30 °C, 190 rpm. The reactions were continued until the substrate was consumed completely.
[b]The reaction was carried out in Tris/HCl buffer (0.3 M, pH 8.5).

Exactly how adding organic solvents in a 10% volume ratio to aqueous medium affects the reaction yield and yeast's enantioselectivity was examined. The solvents were characterized by Log P value, which denotes the hydrophobicity of the organic solvents [17]. The reaction yields were high when petroleum ether or cyclohexane was used, while the other solvents markedly decreased the reaction

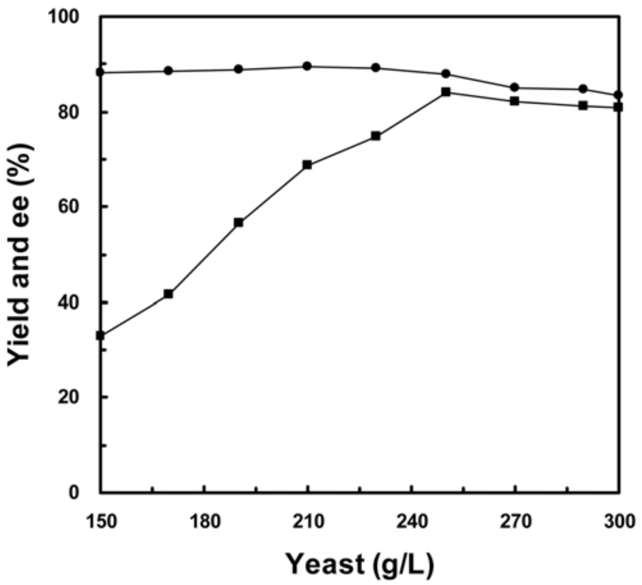

Fig. 1. Effect of yeast concentration on the reduction of ECA. (■), Reaction yield; (●), product's ee. The reaction solution contained 0.54 g glucose, 18.0 mL Tris/HCl buffer (0.3 M, pH 8.5), 2.0 mL petroleum ether, 73.5 mM ECA and various concentrations of wet yeast cells. The reactions were carried out at 30 °C, 190 rpm for 2 h.

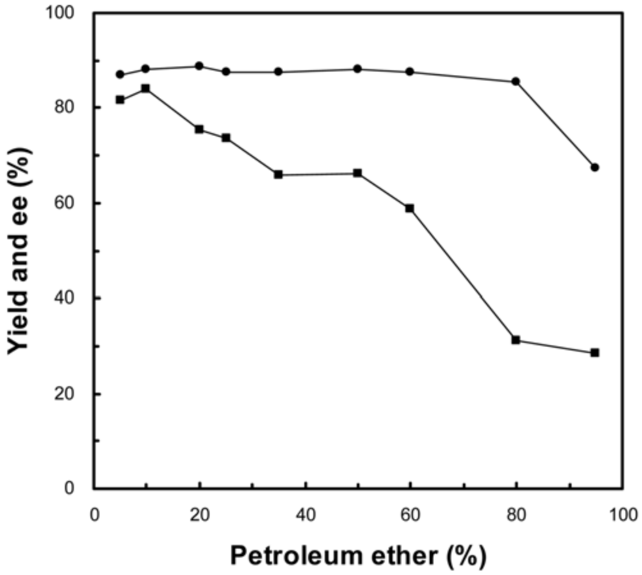

Fig. 2. Effect of volume ratio of petroleum ether in biphasic system on the reduction of ECA. (■), Reaction yield; (●), product's ee. The reaction solution contained 5.0 g wet yeast cells, 0.54 g glucose, 73.5 mM ECA and various volume ratios of petroleum ether in Tris/HCl buffer (0.3 M, pH 8.5). The reactions were carried out at 30 °C, 190 rpm for 2 h.

yield (Table 1). This experimental finding indicates that solvents in a very narrow hydrophobicity (3.20<Log P<3.52) were appropriate for this reaction. On the other hand, the product's ee differed minimally by using these solvents. Among the solvents, although adding n-butanol increased the ee value to 89.1%, its yield declined to an unacceptable value (12.3%). Therefore, petroleum ether was selected for the following investigations. Petroleum ether is frequently used in yeast-catalyzed asymmetric reduction processes in biphasic systems and organic solvent systems [18-20].

Exactly how yeast concentration in this petroleum ether-Tris/HCl buffer biphasic system affects asymmetric reduction of ECA was examined (Fig. 1). When the yeast concentration was low, the product's ee remained relatively at around 89%. However, reaction yield was low, primarily due to the undesired auto-degradation of ECA in the reaction. An optimum yeast concentration of 250 g/L was thus selected.

Next, the volume ratio effect of petroleum ether in this biphasic system on ECA reduction was then studied (Fig. 2). The product's ee remained at around 88% when the volume ratio ranged between 10-80%, whereas the reaction yield declined dramatically when the solvent concentration increased. Thus, an optimum volume ratio of 10% was selected. Compared with data for the aqueous system (yield =74.5%, product's ee=82.3%), reaction yield and product's ee increased to 84.0% and 88.0%, respectively, in this biphasic system.

2. Substrate Stability

The substrate ECA decomposed chemically in aqueous solution, while the product CHBE was stable in the reaction solution [4,9,10]. In this biphasic system, the effect of ECA decomposition was still observed (Fig. 3). The degradation rate can be expressed as

$$v_d=a[S]^b \tag{1}$$

where v_d, a and b represent the degradation rate, rate constant and the rate order, respectively. The logarithm of both sides of this equation is taken as

$$\log v_d=\log a+b\cdot\log[S] \tag{2}$$

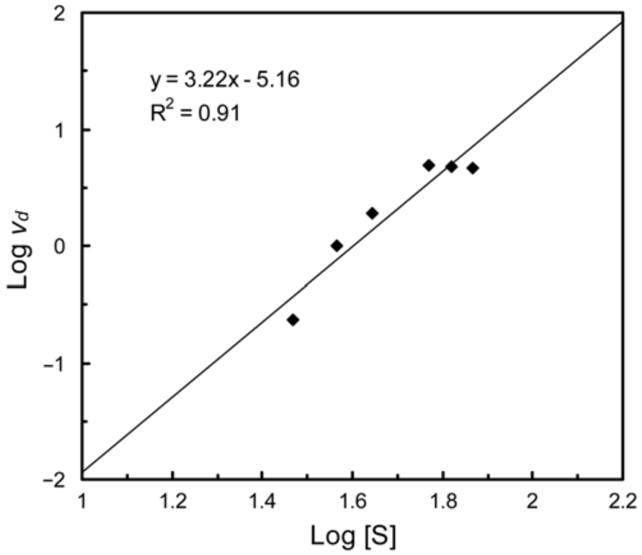

Fig. 4. Plot of log v_d versus log [*S*] of the spontaneous chemical decomposition of ECA in petroleum ether-Tris/HCl buffer biphasic system at 30 °C. The degradation rate v_d was estimated from the initial rate data shown in Fig. 3.

From the plot of log v_d versus log[S] (Fig. 4), the constants a=6.85× 10^{-6} $min^{-1}\cdot mM^{-2.22}$; b=3.22 were estimated. Accordingly, the dependence of the degradation rate on ECA concentration can be expressed as

$$v_d=6.85\times10^{-6}[S]^{3.22} \tag{3}$$

Our previous work reported that the kinetic parameters were a= 7.24×10^{-5} $min^{-1}\cdot mM^{-1.51}$ and b=2.51 in pure aqueous system [15]. The reaction order increased and the rate constant decreased in this biphasic system. This implies that the addition of petroleum ether could significantly decrease the substrate degradation rate.

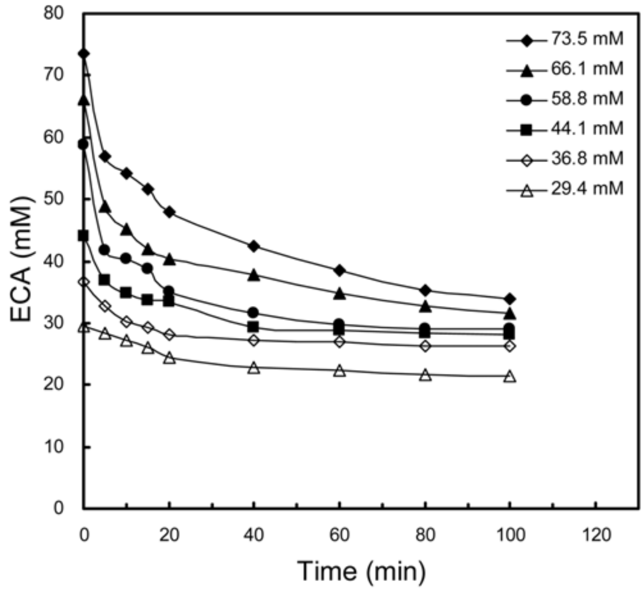

Fig. 3. Spontaneous chemical decomposition of ECA in petroleum ether-Tris/HCl buffer biphasic system at 30 °C. ECA: 73.5 mM (◆), 66.1 mM (▲), 58.8 mM (●), 44.1 mM (■), 36.8 mM (◇), and 29.4 mM (△).

3. Kinetic Behavior of Petroleum Ether-Tris/HCl Buffer Biphasic System

In this reaction, a substrate inhibition effect was found, while no product inhibition was observed. The double reciprocal form of rate equation can be expressed as [21-23]:

$$1/v=\frac{K_m}{V_m}\frac{1}{[S]}+\frac{1}{V_m}+\frac{[S]}{V_mK_i} \tag{4}$$

At low substrate concentration range, Eq. (4) can be simplified to

$$1/v=\frac{K_m}{V_m}\frac{1}{[S]}+\frac{1}{V_m} \tag{5}$$

For such a reaction system, the substrate itself behaves as an inhibitor when its concentration is over a certain high level, then the reaction rate decreases gradually when the substrate concentration is increased. Therefore, there is a maximum point in the plot of reaction rate against substrate concentration. The substrate concentration of this point can be found by d(1/v)/d(1/[S])=0, thus

$$[S]_i=\sqrt{K_mK_i} \tag{6}$$

Therefore, once the K_m value is determined, the value of inhibition constant K_i can then be obtained.

Because the yeast cells contain various reductive enzymes, a complicated model and calculation is required to simulate the overall reaction kinetics with the integration of individual intrinsic kinetics of all related enzymes. For simplifying the simulation process, the system was analyzed with the apparent properties of the whole cell. The apparent kinetic behavior resulting from the various S-enzymes was treated as an enzyme. Its apparent kinetic constants were expressed as V_{mS}, K_{mS} and K_{iS}, while the kinetic constants for the R-enzymes were expressed in the same way as V_{mR}, K_{mR} and K_{iR}.

Fig. 5 shows the Lineweaver-Burk plots for overall reaction, S-enzymes and R-enzymes. An inflection point occurred due to the substrate inhibition effect. The inflection points for overall reaction, S-enzymes and R-enzymes were found at the concentration of 44.99 mM, 45.27 mM and 42.90 mM, respectively.

According to Eq. (5), the values of V_m and K_m were determined from the intercept and the slope of the line of which extrapolation of the line from the low substrate concentration to Y-axis. The K_i value was derived by using Eq. (6). Table 2 lists the kinetic constants for overall reaction, S-enzymes and R-enzymes; the kinetic

Table 2. Comparison of kinetic constants for ECA bioreduction in biphasic system and in aqueous system

Parameter	Biphasic system*	Aqueous system**
$[S]_i$ (mM)	44.99	58.79
V_m (mM/g cell·min)	7.52	11.66
K_m (mM)	102.04	17.84
K_i (mM)	19.84	193.76
$[S]_{iS}$ (mM)	45.27	58.80
$V_{m,S}$ (mM/g cell·min)	7.74	12.41
K_{mS} (mM)	115.06	29.00
K_{iS} (mM)	17.70	119.23
$[S]_{iR}$ (mM)	42.90	58.80
$V_{m,R}$ (mM/g cell·min)	0.24	0.90
K_{mR} (mM)	32.60	35.91
K_{iR} (mM)	56.46	96.29

*Reaction conditions: 5.0 g wet yeast cells, 0.54 g glucose, 18.0 mL Tris/HCl buffer (0.3 M, pH 8.5), 2.0 mL petroleum ether containing 73.5 mM ECA, 30 ºC, 190 rpm.

**The data were from our previous paper [15].

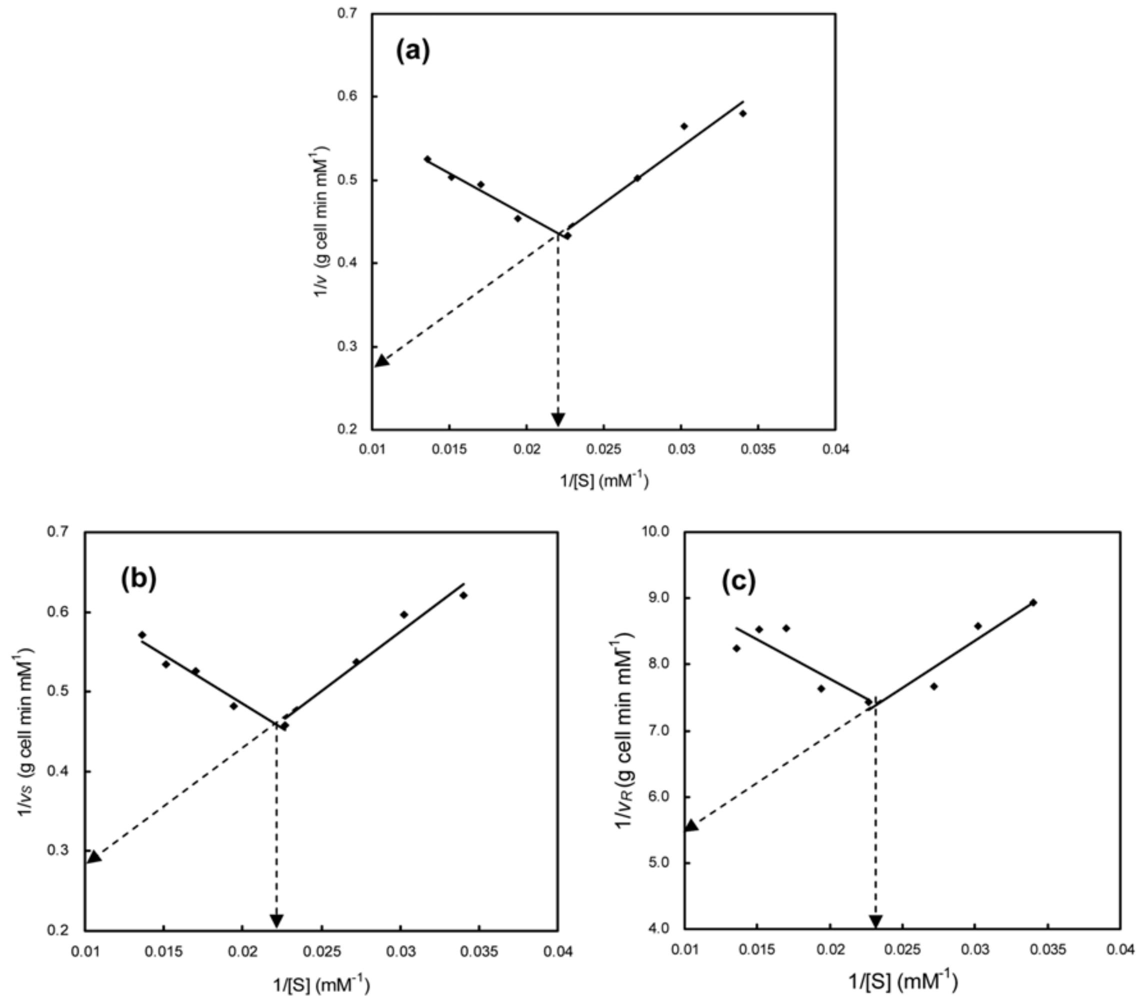

Fig. 5. Lineweaver-Burk plot for (a) overall reaction, (b) S-enzymes and (c) R-enzymes. The reaction solution contained 5.0 g wet yeast cells, 0.54 g glucose, 18.0 mL Tris/HCl buffer (0.3 M, pH 8.5), 2.0 mL petroleum ether and various concentrations of ECA. The reactions were carried out at 30 ºC, 190 rpm.

constants of the same reaction in the aqueous system are also listed for comparison. The $[S]_i$ value of the biphasic system (=44.99 mM) was smaller than that in aqueous system (=58.79 mM). Thus, the substrate inhibition effect occurred at a lower substrate concentration in the biphasic system than in the aqueous system. Moreover, the V_m value of the biphasic system (=7.52 mM/g cell·min) was lower than that of the aqueous system (=11.66 mM/g cell·min). This finding suggests that adding petroleum ether reduced the reaction rate dramatically. For reactions in both systems, the V_m of the S-enzymes was higher than that of R-enzymes. Therefore, the S-CHBE dominated the product solution.

The decrease in yeast's reactive activity when adding organic solvent in the biphasic bioreduction system has been found in several studies [6,13,14]. Living yeast cells are typically harmed when in contact with hydrophobic organic solvents during catalytic processes. Leon et al. [24] reported that organic solvent might cause inactivation or denaturation of membrane-bound proteins, disrupting important transport mechanisms and, at high concentrations, causing cell lysis. Comparing the V_m values reveals that the damage caused by the organic solvent to the R-enzymes was significantly more severe than that of S-enzymes. This finding likely explains why the product's ee of the biphasic system was higher than that of the aqueous system.

Comparing the K_m values, including overall reaction and S-enzymes, for these two systems reveals that the affinities between enzymes and substrate in the biphasic system were both considerably lower than those in the aqueous system. However, the R-enzymes retained an affinity similar to that in the aqueous system. Conversely, all K_i values of the biphasic system were lower than those of the aqueous system, illustrating that the affinity between enzyme and enzyme-substrate complex of yeast cells in the biphasic system was much higher than that of the aqueous system. This high affinity increased the substrate inhibition effect. Moreover, the K_i values of S-enzymes and R-enzymes indicate that the substrate inhibition effect was greater in S-enzymes than in R-enzymes in the biphasic system.

4. Simulation of the Batch Reaction

The modeling and simulation for a reaction system can help us to identify its detailed mechanism. Any significant differences between the system performance and the behavior predicted by the model lets us know that there may be some other important effects that have not been considered.

The substrate consumption is mainly due to the asymmetric reduction catalyzed by baker's yeast and the chemical decomposition of the substrate. In addition, the substrate inhibition effect is also considered. Thus, the rate equation of substrate consumption can be expressed by

$$-\frac{d[S]}{dt}=\frac{V_m[S]}{K_m+[S]+\frac{[S]^2}{K_i}}\times B+a[S]^b \tag{7}$$

where B is the total biomass in the reaction solution. The product CHBE is very stable in aqueous solution. Therefore, the rate of product formation is approximately equal to the bioreduction rate:

$$\frac{d[P]}{dt}=\frac{V_m[S]}{K_m+[S]+\frac{[S]^2}{K_i}}\times B \tag{8}$$

where [P] is the concentration of total products (including S- and R-CHBE). Correspondingly, the rate equation for the formation of S-, R-CHBE and the consumption of substrate can be expressed as

$$\frac{d[P_S]}{dt}=\left(\frac{V_{mS}[S]}{K_{mS}+[S]+\frac{[S]^2}{K_{iS}}}\right)\times B \tag{9}$$

$$\frac{d[P_R]}{dt}=\left(\frac{V_{mR}[S]}{K_{mR}+[S]+\frac{[S]^2}{K_{iR}}}\right)\times B \tag{10}$$

$$\frac{d[P]}{dt}=\left(\frac{d[P_S]}{dt}+\frac{d[P_R]}{dt}\right)\times B \tag{11}$$

$$-\frac{d[S]}{dt}=\left(\frac{d[P_S]}{dt}+\frac{d[P_R]}{dt}\right)\times B+a[S]^b \tag{12}$$

The subscripts S and R in the parameters V_m, K_m and K_i represent the kinetic constants for S- and R-enzymes. $[P_S]$ and $[P_R]$ are the concentration of S- and R-CHBE, respectively. $[S_0]$ is the initial substrate concentration.

Using the kinetic data (Table 2), the formation of CHBE can be predicted by solving Eqs. (7) and (8) simultaneously with the method of Runge-Kutta using Matlab software (Version 5.2.0, Mathworks Co., Natick, MA, USA). According to Fig. 6a, although there are some deviations between the experimental data with simulated values at high substrate concentration during the early phase of the reaction time course, the close correspondence (R=0.9769-0.9986) demonstrates that the mathematical model reasonably represented this biphasic system.

The formation of S- and R-CHBE was simulated by solving Eqs. (9)-(12). As shown in Fig. 6b and 6c, simulation results correlated well with experimental data under different substrate concentrations (R=0.9623-0.9993). The calculated reaction yield and the product's ee values were also in good agreement (R=0.9753 and 0.9995, respectively) with experimental results (Fig. 6d). The reaction yield was >90% when substrate concentration was <55 mM, but decreased as substrate concentration increased. The product's ee did not change significantly under the range of substrate concentrations tested. Thus, when the substrate concentration was reduced from 73.5 mM to 33.1 mM, reaction yield increased from 84.0% to 94.9% with a similar ee value.

CONCLUSIONS

This study demonstrates that in the asymmetric reduction of ECA catalyzed by baker's yeast, adding 10% petroleum ether to the aqueous medium enhances the reaction yield and the product's ee. Kinetic analysis indicates that the characteristics of yeast's enzymes in the aqueous environment were dramatically altered by substituting a portion of water with organic solvent. The yeast's activity declined dramatically and an increased substrate inhibition effect was observed in the petroleum ether-Tris/HCl buffer biphasic system. Nevertheless, a significantly higher ratio for producing S-CHBE compared with that for producing R-CHBE and an enhanced substrate stability in the biphasic medium largely contributed to enhanced reac-

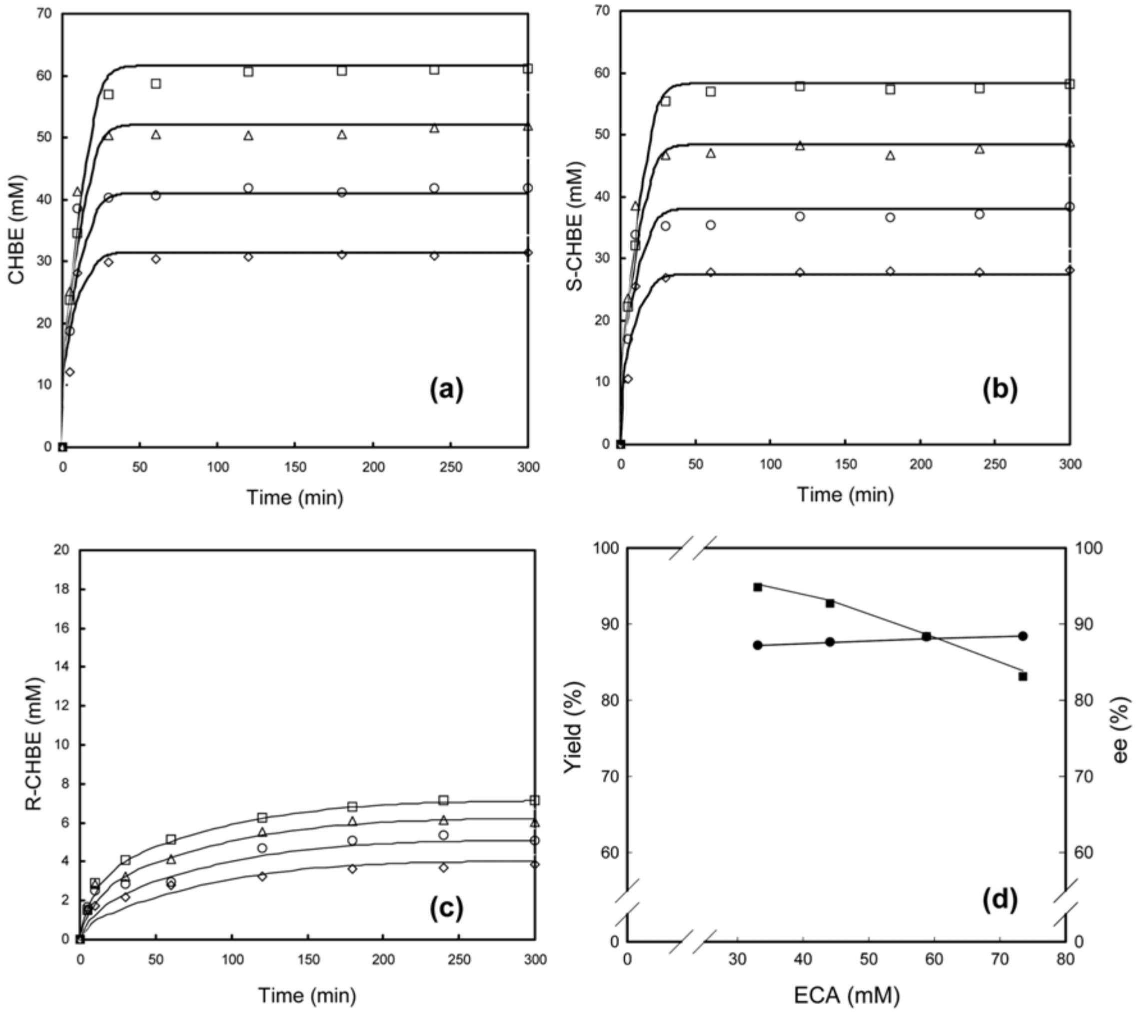

Fig. 6. Comparison of the theoretical prediction values with the experimental results of the ECA bioreduction: (a) CHBE formation, (b) S-CHBE formation, (c) R-CHBE formation, and (d) reaction yield (■) and product's ee (●). Substrate concentration: 73.5 mM (□), 58.8 mM (△), 44.1 mM (○), 33.1 mM (◇). Simulation result: (—). The bioreduction reactions were carried out at 30 °C with 250 g/L yeast cells in petroleum ether-Tris/HCl buffer biphasic solution.

tion performance.

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NOMENCLATURE

a : rate constant of the ECA degradation
b : rate order of the ECA degradation
B : total biomass in reaction solution
K_m : Michaelis-Menten constant [mM]
K_{mR} : Michaelis-Menten constant for R-enzymes [mM]
K_{mS} : Michaelis-Menten constant for S-enzymes [mM]
K_i : inhibition constant [mM]
K_{iR} : inhibition constant for R-enzymes [mM]
K_{iS} : inhibition constant for S-enzymes [mM]
[P] : product concentration [mM]
$[P_S]$: S-CHBE concentration in reaction solution [mM]
$[P_R]$: R-CHBE concentration in reaction solution [mM]
R : correlation coefficient
[S] : substrate concentration [mM]
$[S_0]$: initial substrate concentration [mM]
$[S]_i$: substrate concentration at the inflection point [mM]
t : reaction time [min]
v : reaction rate [mM g cell^{-1} min^{-1}]
v_d : initial degradation rate of ECA [mM min^{-1}]
V_m : maximal reaction rate [mM g cell^{-1} min^{-1}]
V_{mR} : maximal reaction rate for R-enzymes [mM g cell^{-1} min^{-1}]
V_{mS} : maximal reaction rate for S-enzymes [mM g cell^{-1} min^{-1}]

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