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Kinetic model of asymmetric reduction of 3-oxo-3-phenylpropionic acid ethyl ester using *Saccharomyces cerevisiae* CGMCC No.2266

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Abstract−The kinetic model of asymmetric reduction of 3-oxo-3-phenylpropionic acid ethyl ester using *Saccharomyces cerevisiae* CGMCC No.2266 with 10% glucose as co-substrate to realize the regeneration of NADPH was established. The effect factors on reduction, the type and the content of co-substrate and coenzyme, and the changes of the substrate and product content vs. time during the reaction process were investigated. The results indicate that 10% glucose can increase the reaction conversion from 23.0% to 98.4% and NADPH is reducer. The reduction process conforms with sequence mechanisms. The model parameters are as follows: $v_m=5.0\times10^{-4}$ mol·L^{-1}·h^{-1}, $k_1=1.5\times10^{-6}$ mol·L^{-1}·h^{-1}, $k_2=3.0\times10^{-3}$ mol·L^{-1}·h^{-1}. The kinetic model is in good agreement with the experimental data.

Key words: *Saccharomyces cerevisiae* CGMCC No. 2266, Biotransformation, 3-Oxo-3-phenylpropionic Acid Ethyl Ester, (S)-3-hydroxy-3-phenylpropionate, Kinetic Model

INTRODUCTION

(S)-3-hydroxy-3-phenylpropionate is an important intermediate in the synthesis of (S)-fluoxetine, which is a kind of anti-depressant drug [1-4]. It can be gained from the asymmetric reduction of 3-oxo-3-phenylpropionic acid ethyl ester by microbial cells which containing carbonyl reductase as biocatalysts [5-10]. Production of (S)-3-hydroxy-3-phenylpropionate with biotransformation technology is a scientific, economic, and environmentally friendly synthetic method [11-14]. But the current study of the synthesis of (S)-3-hydroxy-3-phenylpropionate by biotransformation remains at a level of laboratory research, and to make it industrialized, it is necessary to establish a kinetic model of the reaction. Mathematical modelling and simulation, supported by detailed kinetic experimentation, offers an important strategy for enhancing productivities and conversions of enzymatic and microbial reduction processes. The establishment of reduction kinetic model has great importance and practical value on the study of microbial reduction conditions, the reaction operation and optimization [15-18].

MATERIALS AND METHODS

1. Microorganism

Saccharomyces cerevisiae CGMCC No. 2266 was gained from the soil in the vicinity of Hang Zhou West Lake Brewery, and it was preserved in the general microbiology center Microbial Culture Collection Management Committee of China on November 26, 2007.

2. Medium

The medium for microorganism preservation was composed of 10 g/L malt juice, 3 g/L yeast extract, 5 g/L peptone, 10 g/L glucose and 20 g/L agar.

The medium for microorganism cultivation was composed of 30 g/L glucose, 3 g/L yeast extract, 5 g/L $(NH_4)_2SO_4$, 0.25 g/L $MgSO_4$, 1 g/L $K_2HPO_4{\cdot}3H_2O$ and 1 g/L KH_2PO_4.

3. Reagents and Instruments

3-Oxo-3-phenylpropionic acid ethyl ester and (S)-3-hydroxy-3-phenylpropionate were purchased from Jiang Su Man Shi Group and J & K Chemical Limited, respectively. Agilent HPLC 1200 was adopted for analysis of conversion and the enantiomeric excess of product. Daicel OB-H chiral column was purchased from Daicel Company.

4. Cultivation of *Saccharomyces cerevisiae* CGMCC No. 2266

The yeast cells picked from the slant were inoculated into 100 ml liquid culture medium and cultivated in a 30 °C shaker (200 r/min) for 24 h. And 10 ml CGMCC No. 2266 gained above was inoculated into 100 ml liquid culture medium. After being cultivated for 24 h, the CGMCC No. 2266 could be used for biotransformation.

5. Reduction by Preheated Yeast Cells

The cultivated cells were separated from culture medium by centrifugation. The sedimentation containing cells was washed twice by sterile water. The cells were dispersed in 20 ml phosphate buffer solution (pH 7.0). After being preheated at 50 °C for some time, the yeast cells could be used for biotransformation. The biomass was 86 g/L (dry weight/reaction volume) and the substrate concentration was 3.63 mmol/L. The reduction happened at 30 °C and 200 r/min.

6. Reduction at Different Temperatures

After yeast suspension was preheated at 50 °C for 30 min, the cells were used for transformation, and the biomass was 86 g/L (dry weight/reaction volume). Substrate concentration was 3.63 mmol/L. The reduction happened at different temperatures and 200 r/min for 24 h.

7. Reduction at Different Reaction Time

After the yeast suspension was preheated at 50 °C for 30 min, CGMCC No. 2266 cells were used for transformation and the biomass was 86 g/L (dry weight/reaction volume). Substrate concentration was 3.63 mmol/L. The reduction happened at 30 °C and 200 r/

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min for some time.

8. Reduction at Different Substrate Concentrations

After the yeast suspension was preheated at 50 ºC for 30 min, the cells were used for transformation, and the biomass was 86 g/L (dry weight/reaction volume). The reduction started when substrate was added at different concentrations. The reduction happened in shaker (200 r/min) at 30 ºC for 24 h.

9. Reduction at Different Biomass

Different amounts of cells were dispersed in pH 7.0 phosphate buffer solution, and preheated at 50 ºC for 30 min. The substrate concentration was 3.63 mmol/L. The reduction happened at 30 ºC and 200 r/min for 24 h.

10. Reduction at Different Co-substrates

Methanol, ethanol, glycerol, butanol and glucose were selected to study the effect of co-substrates on reduction. 86 g/L (dry weight/reaction volume) cells were dispersed in pH 7.0 phosphate buffer solution. After the yeast suspension was preheated at 50 ºC for 30 min, substrate and co-substrate were added into it. The reduction happened at 30 ºC and 200 r/min for 24 h.

11. Analytical Method

When the reduction ended, hexane was used to extract the substrate and product in the supernatant fluid from the centrifugation of the reaction mixture. The hexane layer containing substrate and product was detected by Agilent HPLC 1200. Daicel OB-H chiral column was put in the 35 ºC oven. The mobile phase consists of hexane and isopropanol (90 : 10). The flow rate of mobile phase was 1.0 ml/min. The UV detection wavelength was 220 nm.

NADH and NADPH concentration were detected after cell breaking according to the reports of Bubis [19] and Antonella [20].

12. Data Processing

The conversion was calculated by Eq. (1). M_S and M_P stand for the molecular weight of substrate and product. Q and P stand for initial substrate weight and product weight at the end of the reaction.

$$\text{Conversion} = \frac{O}{\frac{M_P}{M_S} \times Q} \times 100\% \quad (1)$$

The enantiomeric excess of product was calculated by Eq. (2). C_R and C_S stand for (R)-3-hydroxy-3-phenylpropionate and (S)-3-hydroxy-3-phenylpropionate conc-entration.

$$\text{The enantiometric excess of product} = \frac{C_S - C_R}{C_S + C_R} \times 100\% \quad (2)$$

RESULTS AND DISCUSSION

1. Influence of Preheat Treatment of Yeast Cells on Reduction

The carbonyl reductase in the yeast cell was composed of R-reductase and S-reductase. R-reductase and S-reductase can reduce the substrate to (R)-3-hydroxy-3-phenylpropionate and (S)-3-hydroxy-3-phenylpropionate, respectively. Preheat treatment of yeast cells can reduce the activity of carbonyl reductase. Table 1 shows that the conversion decreased and the enantiomer excess of (S)-3-hydroxy-3-phenylpropionate increased with the extension of preheat time. The content of S-reductase in the yeast cell was more than that of R-reductase. When the yeast cells were preheated for some time, R-reductase lost catalytic activity so that the enantiomer excess of (S)-3-hydroxy-3-phenylpropionate increased. When the yeast cells were used in reduction after being preheated at 50 ºC for 30 min, the enantiomer excess of (S)-3-hydroxy-3-phenylpropionate was over the detection limit (e.g., 99.5%).

Table 1. Effect of preheat treatment of CGMCC No. 2266 on reduction

Preheating time (min)	Conversion (%)	Enantiomer excess value of (S)-3-hydroxy-3-phenylpropionate (ee%)
0	27.0	97.0
5	26.3	97.2
10	25.8	98.5
15	25.1	98.8
20	24.5	99.2
25	23.7	99.7
30	23.0	Over the detection limit (ex. 99.5%)
35	21.8	Over the detection limit (ex. 99.5%)

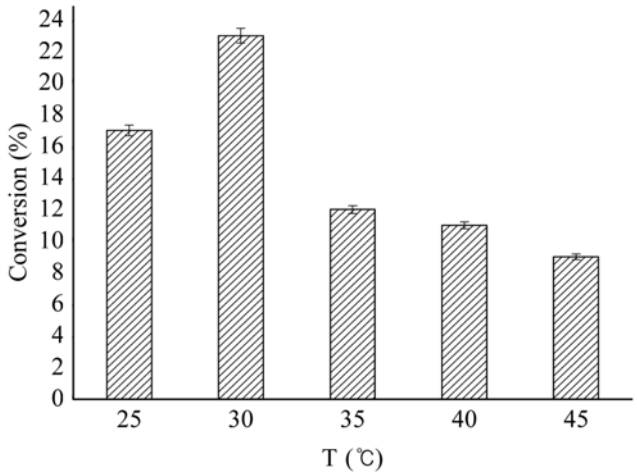

Fig. 1. Effect of temperature on the formation of (S)-3-hydroxy-3-phenylpropionate (Substrate concentration 3.63 mmol/L, biomass 86 g/L (w/v)).

2. Influence of Temperature on Reduction

The yeast cells have different reduction activity at different temperatures, and higher temperature can accelerate the inactivation rate of carbonyl reductase. Fig. 1 shows that the optimum temperature was 30 ºC. The enantiomer excess of (S)-3-hydroxy-3-phenylpropionate was all over the detection limit (e.g., 99.5%) at different temperatures.

3. Influence of Reaction Time on Reduction

Fig. 2 shows the optimal reaction time was 24 h. The enantiomer excess of (S)-3-hydroxy-3-phenylpropionate did not change with reaction time, and was all over the detection limit (e.g., 99.5%).

4. Influence of Substrate Concentration on Reduction

Fig. 3 shows that² the conversion decreased rapidly with the increase of substrate concentration. The high substrate concentration has a poisoning effect on microorganisms and inhibits the activity of carbonyl reductase [21]. The enantiomer excess of (S)-3-hydroxy-3-phenylpropionate was all over the detection limit (e.g.. 99.5%) at

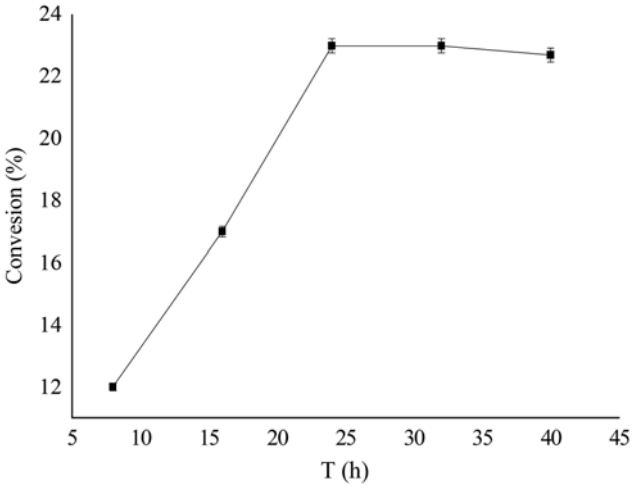

Fig. 2. Time course of reduction (Substrate concentration 3.63 mmol/L, biomass 86 g/L (w/v)).

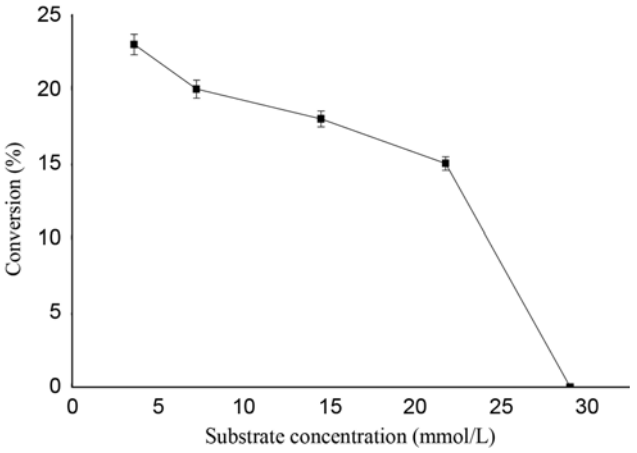

Fig. 3. Effect of substrate concentration on reduction (Biomass 86 g/L (w/v)).

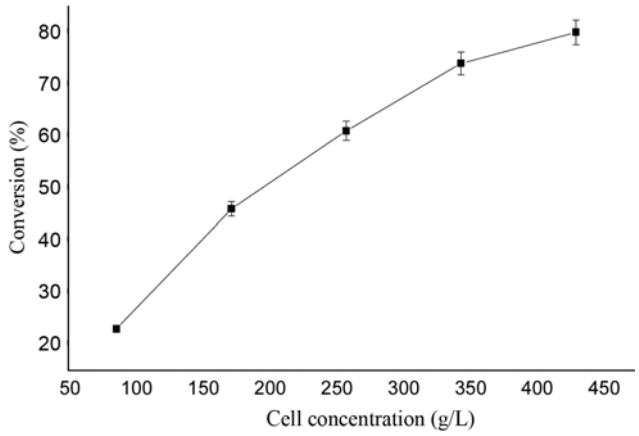

Fig. 4. Effect of biomass on reduction (Substrate concentration 3.63 mmol/L).

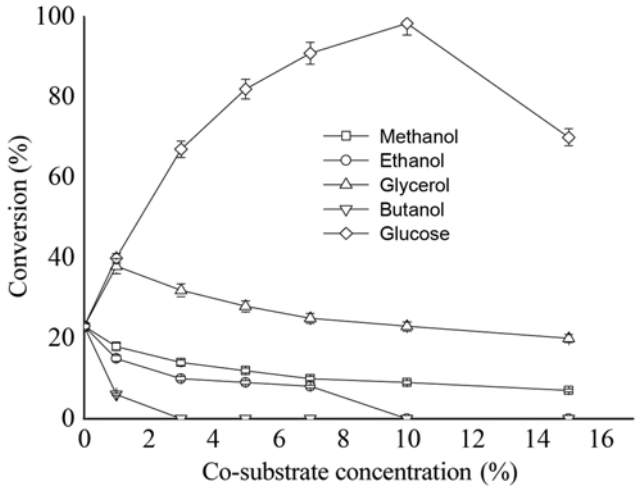

Fig. 5. Effect of the co-substrates on the formation of (S)-3-hydroxy-3-phenylpropionate.

different substrate concentration.

5. Influence of Biomass on Reduction

Fig. 4 shows the conversion increased with the increase of biomass. The conversion reached 80% when the biomass was 430 g/L (dry weight/reaction volume). The quantity of enzyme and the concentration of NADH or NADPH increased with addition of more biomass so that the conversion improved. The enantiomer excess of (S)-3-hydroxy-3-phenylpropionate did not change when the biomass changed, and was all over the detection limit (e.g., 99.5%).

6. Influence of Co-substrate on Reduction

Methanol, ethanol and butanol cannot improve the conversion, while glycerol and glucose behave better. With glucose and glycerol as co-substrate, the conversion improved greatly. The conversion can reach 98.4% with 10% glucose as co-substrate. Yeast cells contain a large amount of glucose dehydrogenase, which can convert glucose to gluconic acid, and the hydride from glucose transfers to $NADP^+$ to form NADPH, which supplies the hydrogen in reduction [22]. The co-substrate glucose can help the regeneration

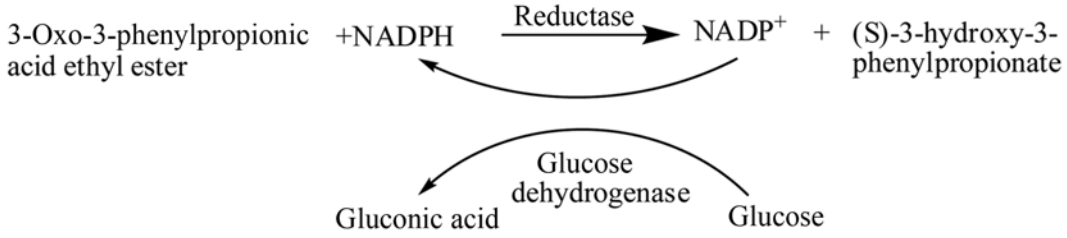

Fig. 6. Reduction of 3-oxo-3-phenylpropionic acid ethyl ester with coenzyme regeneration.

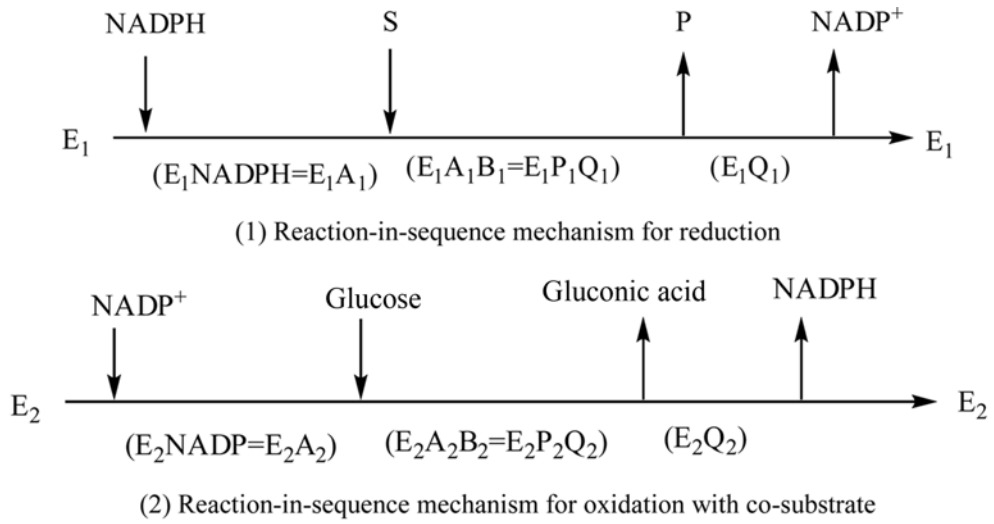

Fig. 7. Reaction-in-sequence mechanisms for two oxidation-reduction reactions.

of NADPH in cells and improve the conversion.

The reduction of 3-oxo-3-phenylpropionic acid ethyl ester by CGMCC No. 2266 with coenzyme regeneration can be regarded as a coupling of two reactions: the reduction of the substrate which consuming NADPH and the oxidation of the co-substrate with regeneration of NADPH. Figure 6 shows the reaction mechanism.

7. The Kinetic Model

It is assumed that the two reactions above are in line with the sequence reaction mechanism, and the mechanisms of the two reactions above are shown in Fig. 7.

The kinetics equation of the oxidation-reduction reaction which conforms with sequence mechanisms can be written as:

$$v = \frac{v_m C_S C_{NADPH}}{k_1 C_{NADPH} + k_1 k_2 + c_S c_{NADPH}} \quad (1)$$

8. Type and Content of Coenzyme Reduction with Glucose as Co-substrate

Oxidoreductase has specificity for coenzyme. Some require NAD (NADH), and some require NADP (NADPH). Table 2 and Table 3 show the content of NADH and NADPH varied with the reaction time in different initial substrate concentration. The NADPH concentration maintained 40 μmol·L^{-1} and changed little with the reaction time. The NADH concentration reduced to zero after 30 min. NADPH was determined to be coenzyme participating in the reduction.

9. Determination of Model Parameters

The NADPH concentration maintains 40.0 μmol/L, which is adopted in formula (1), and formula (1) can be written as:

$$v = \frac{v_m c_s \cdot 40\times10^{-6}}{k_1 \cdot 40\times10^{-6} + k_1 k_2 + c_s \cdot 40\times10^{-6}} \quad (2)$$

$$-dc_s/dt = \frac{v_m c_s \cdot 40\times10^{-6}}{k_1 \cdot 40\times10^{-6} + k_1 k_2 + c_s \cdot 40\times10^{-6}} \quad (3)$$

$$-\left[\frac{k_1}{v_m} + \frac{k_1 k_2}{v_m \times 40\times10-6}\right] \ln c_s \Big|_{c_{s0}}^{c_s} + \frac{1}{v_m} c_s \Big|_{c_{s0}}^{c_s} = t\Big|_0^t + A \quad (4)$$

The kinetic parameters obtained through fitting with experimental data are as follows: v_m=5.0×10^{-4} mol·L^{-1}·h^{-1}, k_1=1.5×10^{-6} mol·L^{-1}·h^{-1}, k_2=3.0×10^{-3} mol·L^{-1}·h^{-1}.

The kinetic model is expressed as follows:

$$v = \frac{5.0\times10^{-4} \cdot c_s \cdot c_{NADPH}}{1.5\times10^{-6} \cdot c_{NADPH} + 1.5\times10^{-6}\times3.0\times10^{-3} + c_s \cdot c_{NADPH}} \quad (5)$$

Table 2. NADPH concentration at different reaction time and different initial substrate concentration

Reaction time (h)	Initial substrate concentration (mmol·L^{-1})				
	1.74	3.47	5.21	6.94	8.68
	NADPH concentration (μmol·L^{-1})				
0	39.9	40.1	40	40	40.2
0.5	40	40.2	40	40.2	40
1	40.1	40	40.1	40	40
6	40.3	40.3	40.2	40.3	40.2
12	40	40	39.9	40	40
18	39.9	40	39.7	40	39.7
24	40.2	40.1	40	40	39.8
30	39.9	40	40	40.1	40

Table 3. NADH concentration at different reaction time and different initial substrate concentration

Reaction time (h)	Initial substrate concentration (mmol·L^{-1})				
	1.74	3.47	5.21	6.94	8.68
	NADH concentration (μmol·L^{-1})				
0	0.51	0.5	0.49	0.5	0.5
0.5	0	0	0	0	0
1	0	0	0	0	0
6	0	0	0	0	0
12	0	0	0	0	0
18	0	0	0	0	0
24	0	0	0	0	0
30	0	0	0	0	0

Table 4. Comparison of experimental data and model data

Initial substrate concentration (mmol/L)		1.740	3.470	5.210	6.740	8.680
Experimental data of product concentration (mmol/L)	0 h	0.000	0.000	0.000	0.000	0.000
	6 h	0.818	1.217	2.041	2.237	2.116
	12 h	1.253	2.391	3.016	3.608	4.438
	18 h	1.536	2.990	4.407	5.116	6.113
	24 h	1.698	3.283	4.814	5.976	7.374
	30 h	1.738	3.410	5.078	6.748	8.000
Simulated data of product concentration (mmol/L)	0 h	0.000	0.000	0.000	0.000	0.000
	6 h	0.873	1.405	1.758	1.991	2.152
	12 h	1.364	2.415	3.173	3.708	4.092
	18 h	1.596	3.001	4.163	5.072	5.749
	24 h	1.694	3.294	4.759	6.012	7.033
	30 h	1.734	3.422	5.052	6.558	7.901
R^2		0.995	0.997	0.993	0.997	0.998

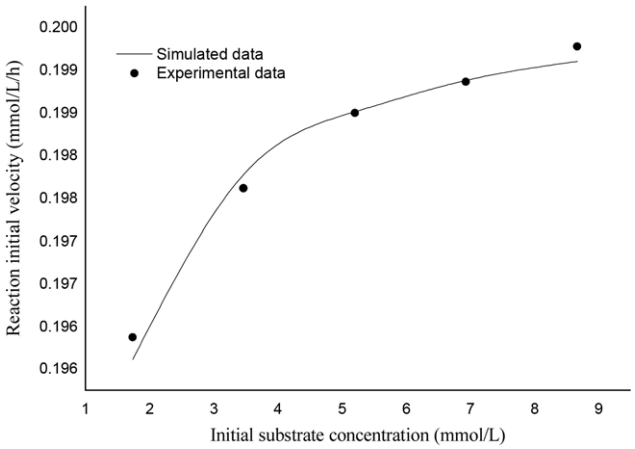

Fig. 8. Initial reaction velocity changes with initial substrate concentration (Biomass 86 g/L (w/v)).

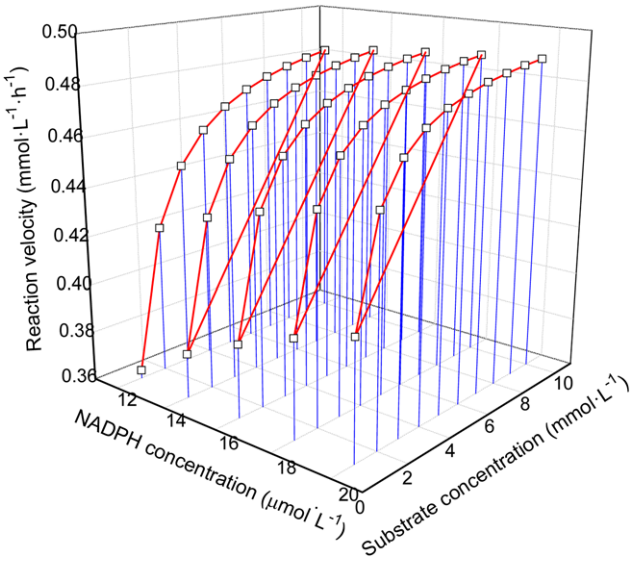

Fig. 9. Reaction velocity changes with NADPH concentration and substrate concentration (Biomass 86 g/L (w/v)).

10. Comparison of Experimental Data and Model Data

Comparison of experimental data and model data is revealed in Table 4. The relationship between the reaction initial velocity and initial substrate concentration is illustrated in Fig. 8. The results show that model data fit well with experimental data. The kinetic model obtained describes the reduction process of 3-oxo-3-phenylpropionic acid ethyl ester by CGMCC No. 2266 with 10% glucose as co-substrate to carry out the regeneration of NADPH.

11. Relationship between Reaction Velocity and the Concentration of NADPH and Substrate

The relationship between reaction velocity and the concentration of NADPH and substrate was indicated by the kinetic model and shown in Fig. 9. When NADPH concentration was constant, the reaction velocity increased with the increase of substrate concentration. When substrate concentration was constant, the reaction velocity increased with the increase of NADPH concentration. For a reversible reaction with two kinds of substrates, the increase of concentration of one of the two substrates will make the reaction move to the direction of product formation, so as to enhance the conversion.

CONCLUSIONS

The pretreatment of cells can improve the enantiomer excess value over the detection limit (e.g., 99.5%). 10% glucose was selected as optimum co-substrate, for it can improve the conversion more greatly than other co-substrates such as methanol, ethanol, glycerol, and butanol. Through the determination of NADH and NADPH concentration in the process of reduction, NADPH was confirmed to participate in the reduction. The model parameters obtained through fitting with experimental data are as follows: $v_m=5.0\times10^{-4}$ mol·L^{-1}·h^{-1}, $k_1=1.5\times10^{-6}$ mol·L^{-1}·h^{-1}, $k_2=3.0\times10^{-3}$ mol·L^{-1}·h^{-1}.

The kinetic equation is expressed as follows:

$$v=\frac{5.0\times10^{-4}c_s c_{NADPH}}{1.5\times10^{-6}c_{NADPH}+1.5\times10^{-6}\times3.0\times10^{-3}+c_s c_{NADPH}}$$

The kinetic model can be fitted well with the experimental data.

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NOMENCLATURE

v : reaction rate
v_m : maximum reaction rate
c_s : substrate concentration
c_{NADPH}: NADPH concentration
k_1, k_2 : the first and second enzymatic reaction rate constant in the reaction

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