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Kinetic analysis of 11α-hydroxylation of steroids by *Rhizopus nigricans*

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ABSTRACT

Optimization of bioconversion of 16,17α-epoxyprogesterone by *Rhizopus nigricans* TJ 108 was investigated by means of uniform design. Batch cell growth and bioconversion kinetics were simulated under optimal conditions. Contois equation was used in the kinetics study of fungal growth on glucose. Simulation of bioconversion process was done at a constant value of substrate concentration according to the reaction mechanism. It was demonstrated that the above model satisfactorily described the kinetic behaviors of cell growth and bioconversion of the filamentous fungi.

1. Introduction

The importance of microbial biotechnology in the production of steroid drugs and hormones was realized for the first time in 1952 when Murray and Peterson of Upjohn Company patented the process of 11α-hydroxylation of progesterone by a *Rhizopus species* [1]. Since then, microbial reactions for the transformation of steroids have proliferated, and gradually replaced the chemical reactions in some key steps in the production of therapeutically useful drugs and hormones.

Filamentous fungus *Rhizopus nigricans* is a well-known hydroxylator of epoxyprogesterone at the position of 11α (Fig. 1), so it is often chosen as a model system when introducing a hydroxyl group into a steroidal ring system in the eukaryotic microorganisms [2,3]. Now many reports on the hydroxylation of steroids by *R. nigricans* had been issued [4,5]. However, there are no literatures about the kinetic model research in steroid bioconversion process. Mathematical modeling and simulation, supported by detailed kinetic experiments, offer an important strategy for enhancing productivities and yields of enzymatic and microbial cultivation processes [6,7].

In the reaction of steroid 11α-hydroxylation, the solubility of the substrate 16,17α-epoxyprogesterone was low, which was about 0.1 mmol/L in water [8]. Generally, in order to conduct the process economically feasible, the substrate was excessive relative to its solubility. In this study, an alternative approach was used that the reaction was carried out in a heterogeneous system in which most of the substrate was present in suspended solid particles [9–11]. In the simulation of the whole bioconversion process, the effects of the substrate solubility, transfer of the dissolved substrate through the cell membrane were neglected for simplification [12].

The statistical methodologies in experimental design of biotechnology processes were increasingly used [13–15]. The conventional optimization of varying one factor at a time and maintaining other variables influencing the process at a constant level does not point out the combined effect of all the process variables, while the statistical methodologies can overcome the disadvantage. Uniform design, one of the space filling designs, explores relationships between the response and the factors so that it can be used for experiments in which a response of interest is influenced by several factors. Uniform design tables can be denoted by $U_n(q^s)$, where U stands for the uniform design, n for the numbers of experimental trials, q for the number of levels and s for the maximum number of factors. The total number of factor level combinations is then q^s, which is too high to be executed using orthogonal design, even when s and q are moderate. However, with a uniform design the number of experiments can be decreased significantly [16,17]. Therefore, the aim of uniform design is to get the optimum conditions with less runs, decreasing the expense and time of experiments. A number of examples of successful application of the uniform design method for improving process have been reported [18,19]. But the uniform design method had not been reported to be used in the 11α-hydroxylation by *R. nigricans*.

The objectives of this study were to use uniform design to optimize the culture medium in an aqueous suspension of a solid substrate system, and then to investigate the cell growth and the

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Nomenclature

C_W	substrate 16,17α-epoxyprogesterone concentration (g/L)
C_X	cell concentration (g/L)
$C_{X,max}$	maxmium cell concentration (g/L)
C_S^0	initial glucose concentration (g/L)
C_X^0	initial inoculation concentration (g/L)
E_0	the initial enzyme (units/g dcw)
k_1	productive constant of [*ES*]
k_{-1}	desorption constant of [*ES*]
k_2	bioconversion constant
k_{-2}	product inhibition constant
K_C	Contois saturation constant
K_m	half saturation coefficient
l_1	the ratio of dissolved substrate in the whole 16,17α-epoxyprogesterone
l_2	the ratio of dissolved product in the whole product
P	product concentration (g/L)
P_t	the product concentration at the time of t (g/L)
[P]	dissolved product concentration inside the cell (g/L)
S_0	the initial 16,17α-epoxyprogesterone concentration (g/L)
S_t	the substrate 16,17α-epoxyprogesterone concentration at the time of t (g/L).
[S]	dissolved substrate concentration in the aqueous phase (g/L)
X_1	coded value of glucose (g/L)
X_2	coded value of corn steep liquor (g/L)
X_3	coded value of $(NH_4)_2SO_4$ (g/L)
X_4	coded value of yeast extract (g/L)
X_5	inoculation (spores/L)
X_6	pH
Y_P	bioconversion rate
Y_{XS}	the cell growth yield coefficient

Greek symbols

α	residual carbon sources concentration (g/L)
μ_m	maximal specific growth rate (h^{-1})
μ_X	specific growth rate (h^{-1})

simple steroid substrate bioconversion kinetics under the optimal conditions.

2. Materials and methods

2.1. Steroids

A powder of 16,17α-epoxyprogesterone ($C_{21}H_{28}O_3$, molecular weight 328.46) was obtained from Tianjin Jinjin Pharmaceutical Co. Ltd., China. Its purity was higher than 99% determined by HPLC. The substrates were filtered through molecular sieve (pore size of 45 μm) before making suspension, which contained: 16,17α-epoxyprogesterone, 1 g; washing powder, 0.035 g; ethanol, 1.5 mL and water, 8 mL.

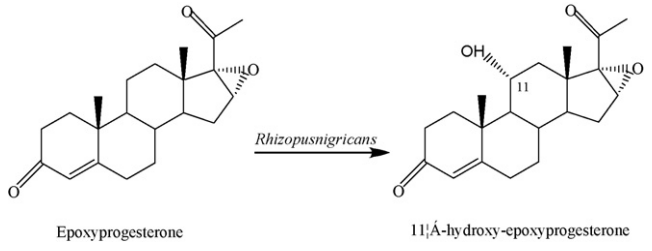

Fig. 1. 11α-Hydroxylation of 16,17α-epoxyprogesterone by filamentous fungus *Rhizopus nigricans*.

2.2. Microorganism and cultivation conditions

R. nigricans TJ 108, obtained from Tianjin Jinjin Pharmaceutical Co. Ltd., China, was used throughout the experiment. Before inoculation of submerged cultures, the fungus was maintained at 28 °C for 7–10 days on agar slants, which contained (g/L): yeast extract, 10; glucose, 15; NaCl, 5; KH_2PO_4, 5; agar, 35; with pH adjusted to 6.5. Liquid cultures were grown in the medium contained (g/L): yeast extract, 0.86; glucose, 25; $(NH_4)_2SO_4$, 0.1; corn steep liquor, 22; with initial pH adjusted to 3.4. 250 mL Erlenmeyer flasks containing 40 mL of growth medium were inoculated with 7–10 days old spores of *R. nigricans* TJ 108 with 1 mL of the initial spore concentrations of about 6.55×10^6 spores/L. Submerged cultivation was performed for 28 h on a rotary shaker at 200 rpm at 28 °C, and then the prepared substrate 16,17α-epoxyprogesterone suspensions (1.75 g/L final concentration) were added in the flasks and cultivated continuously for more 48 h. All experiments were repeated at least three times, and the data shown in the corresponding table and figures in this paper were the mean values of the experiments.

2.3. Uniform design

According to the single factor experiments, bioconversion process of *R. nigricans* TJ 108 was strongly influenced by several fermentation conditions: glucose (X_1), corn steep liquor (X_2), $(NH_4)_2SO_4$ (X_3), yeast extract (X_4), inoculation concentration (X_5) and pH (X_6) [20,21]. The larger the initial range of the experimental factors investigated, the greater the chance for the uniform design to converge to the global optimum [22]. Therefore, the experimental range of the factors was chosen as large as possible for optimization. All factors examined and their experimental ranges investigated were shown in Table 1.

In view of the practical conditions, sixteen levels were selected for all of the six factors. The *U*-type design $U_{16}^*(16^{12})$ was applied. According to the regulation of forming uniform table, the 1, 3, 5, 8, 10, and 11 rows were used to form the $U_{16}^*(16^6)$. The experiments were made under the using request of $U_{16}^*(16^6)$. The next step was then to find a suitable model fitting the experimental data using regression analysis, in order to select the optimum from the model built [23,24]:

$$Y_P = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where Y_P was the dependent variable (bioconversion rate), β_0 the interception coefficient, β_i the linear terms coefficients, β_{ii} the quadratic terms coefficients, β_{ij} the interaction terms coefficients, and x_i, x_j were the factors. This expression described the interaction of the various independent variables on the value of bioconversion

Table 1
Factors investigated and their experimental range

Factors	Experimental range
X_1, glucose (g/L)	10–40
X_2, corn steep liquor (g/L)	10–40
X_3, $(NH_4)_2SO_4$ (g/L)	0.1–3.1
X_4, yeast extract (g/L)	0.1–3.1
X_5, inoculation (spores/L)	5×10^5–1.55×10^7
X_6, pH	2.8–5.8

Table 2
The output results of stepwise regression

		Step							
		1		2		3		4	
Factors	Constant	X_{66}	$P>F$	X_{34}	$P>F$	X_6	$P>F$	X_{16}	$P>F$
Value	0.03	0.056	<0.0001	0.024	<0.0001	0.33	0.0001	1.3×10^{-3}	0.001

	Step							
	5		6		7		8	
Factors	X_{11}	$P>F$	X_{46}	$P>F$	X_4	$P>F$	X_{22}	$P>F$
Value	2.2×10^{-4}	0.001	0.019	0.0004	−0.049	0.007	1.4×10^{-4}	0.003

	Step								
	9		10		11		12		
Factors	X_{12}	$P>F$	X_{55}	$P>F$	X_{15}	$P>F$	X_{44}	$P>F$	R-square
Value	2.4×10^{-4}	0.005	-4.5×10^{-16}	0.014	2.3×10^{-10}	0.025	−0.008	0.045	0.9999

rate. The Fish's test was performed on experimental data to evaluate the statistical significance of the model. If the regression effect is very good, then 1stOpt soft (Data Processing System, China) was used to obtain the maximum of the second-order polynomial. And the values of factors corresponding to the maximum were useful.

2.4. Analytical methods

Cell concentration on a dry basis were measured by filtering the filamentous cell with a filter, washing with distilled water to remove the residual culture medium and drying constant weight for 24 h at 95 °C. Glucose concentration in the medium was determined by the glucose–lactic acid biosensor (Shandong Institute of Bioengineering, China; Type SBA). Reaction products were extracted from the cell by circumfluence with acetone because they were existed inside the cell, and quantitatively evaluated by HPLC using a LabAlliance (model Series III) system, with a C18 column (250 mm × 4.6 mm, LabAlliance, USA). Elution was performed with 325/125/50 (v/v) methanol/water/acetonitrile at a flow rate of 1.0 mL/min, and detection was realized with a UV detector (Model 500, LabAlliance, USA) at 254 nm.

3. Results and discussion

3.1. Optimization of the medium

A $U^*_{16}(16^6)$ experimental design was used to determine the relationships existing between the components of the medium. The following equation calculated for maximum bioconversion rate was obtained from the experimental results using the stepwise regression of SAS 9.0 software, after eliminating the statistically insignificant terms:

$$\begin{aligned} Y_P = {} & 0.03 - 0.049\times x_4 + 0.33\times x_6 - 2.2\times10^{-4}\times x_1^2 \\ & -1.4\times10^{-4}\times x_2^2 - 8.2\times10^{-3}\times x_4^2 \\ & -4.5\times10^{-16}\times x_5^2 - 0.056\times x_6^2 + 2.4\times10^{-4}\times x_1\times x_2 \\ & +2.3\times10^{-10}\times x_1\times x_5 \\ & +1.3\times10^{-3}\times x_1\times x_6 - 0.024\times x_3\times x_4 + 0.02\times x_4\times x_6 \end{aligned} \quad (2)$$

The analysis of variance (ANOVA) $P<0.0001$ and value of F test of the model ($F=2056.57$) showed that the model was actually significant at 99% confidence level. Besides, the fit of the model was checked by the coefficient of determination (R^2), which was found to be 0.9999, which demonstrated a good correlation between the independent variables. According to the principle of the stepwise, the earlier the factor was introduced in the model, the more important the factor was [20]. It was seen from Table 2 that X_{66} was first one introduced in the model. So pH (X_6) was the most important factor. Furthermore, $P<0.0001$ of X_{66} also approved this. In Table 2, P of every factor was less than 0.05 showing that these factors were all significant terms at this level.

The maximum of the above function was 0.562 at the $X_1=25$ (g/L), $X_2=22$ (g/L), $X_3=0.1$ (g/L), $X_4=0.86$ (g/L), $X_5=6.55\times10^6$ spores/L and $X_6=3.4$ which were obtained using 1stOpt software with Levenberg–Marquardt method.

To verify the feasibility of the uniform design, five repeat experiments were made. The mean result of verifying experiments was 0.559 (%R.S.D. < 2), which was very close to the predicted value. This result was higher than that of Mao et al. [25], which was 0.53.The regression function can show every factors response to the bioconversion rate.

3.2. Fermentation process

In order to study the cell growth process at different glucose concentrations, experiments with glucose concentration from 0 to 30 g/L at the interval of 5 g/L were conducted. After a lag phase, cell concentration and glucose concentration were monitored at the same time and same intervals. Fig. 2 shows cell growth and the glucose consumption at the glucose concentration of 0–30 g/L with the same 1 mL of initial inoculation concentration of 6.55×10^6 spores/L and same cultivation conditions. Cell grew after 10 h and the cell yields were in accordance with the

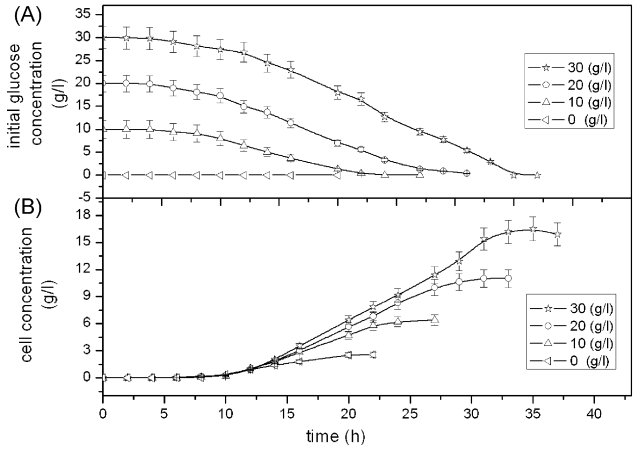

Fig. 2. Time curves of glucose consumption (A) and cell growth (B) by *Rhizopus nigricans* TJ 108 with the initial glucose concentration from 0 to 30 g/L.

step increase of initial glucose concentration. The measurements of total nitrogen concentration revealed an excess of this substrate during cultivation and had no limiting effect on the growth of *R. nigricans* TJ 108 (data not present). According to the above consideration, it was concluded that there was no glucose inhibition effect on the cell growth. What is more, from Fig. 2(B), it was seen that the cell concentration of the filamentous fungus was very high. Taking the initial glucose concentration of 20 g/L for example, its corresponding cell concentration was about 11 g/L.

3.3. Kinetics study

3.3.1. Growth kinetics study

In most fermentation processes, it is widely recognized that optimal design and control of fermentation systems requires quantitative estimates of microbial growth, substrate consumption [26]. The first step in modeling a fermentation process is the selection of a proper mathematical expression, which describes microbial growth. In this experiment, batch culture of *R. nigricans* TJ 108 was conducted in the medium under the substrate glucose as the main carbon source with its initial concentration ranging from 0 to 30 g/L. The specific cell growth rates were calculated as follows:

$$\frac{dC_X}{dt} = \mu_X C_X \tag{3}$$

where μ_X was the specific growth rate of *R. nigricans* TJ 108 (h^{-1}). C_X was the cell concentration (g/L).

Among the equations describing microbial growth kinetics the Monod equation has been widely used in the literatures. It describes the relationship between the microbial specific growth rate and the limiting substrate concentration using the following form:

$$\mu_X = \frac{\mu_m C_S^0}{K_m + C_S^0} \tag{4}$$

where the constant μ_m and K_m were the maximal specific growth rate and the half saturation coefficient, respectively, for a given microbe–substrate system.

While for filamentous microorganisms' fermentation, it has been proposed that the cell concentration also had effect on the specific growth rate besides the limiting substrate concentration [27]. These fermentations are often characterized by high cell concentrations [28,29]. High cell concentrations, coupled with the possibility of hyphal entanglement, adversely impact the diffusion of nutrients and oxygen [30]. Bajpai and Reub [26] suggested that these diffusional limitations could manifest as an increase in the apparent value of the half saturation coefficient K_m with increased cell concentrations. Thus, the Contois equation [31] may be used to describe such diffusion-limited microbial growth as it assumed the specific growth rate to be a function of both microbial and limiting substrate concentrations as:

$$\mu_X = \frac{\mu_m C_S^0}{K_C C_X + C_S^0} \tag{5}$$

where K_C was the Contois saturation constant and C_S^0 was the initial glucose concentration. This equation had already been used successfully in describing fungal growth kinetics such as *Penicillium chrysogenum* [32].

In this experiment when there was no glucose in the culture, it still had unexpected microbial growth (Fig. 2). May be there were some other carbon sources besides glucose in the yeast extract or corn steep liquor. In this experiment, the concentrations of yeast extract and corn steep liquor were constant at different initial glucose concentration. The modified Contois equation was used:

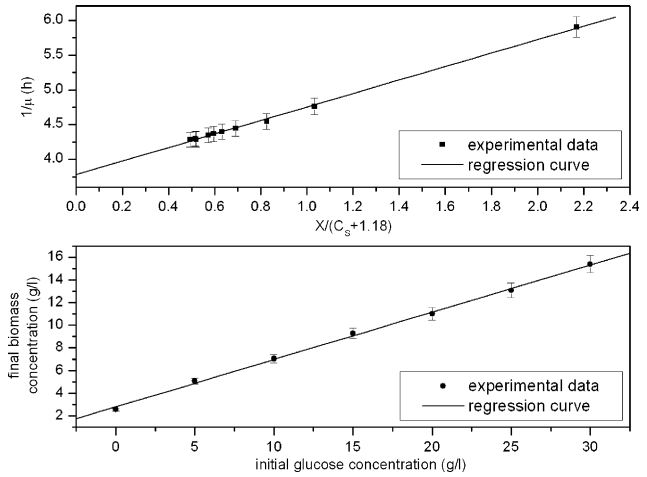

Fig. 3. (A) Linear plot from the specific growth rate and the ratio of the cell concentration vs. initial glucose concentration. The intercept is 3.78287, the slop is 0.97052, and the R^2 is 0.9986. (B) Linear plot from cell concentration and initial glucose concentration. The slop is 0.42061, and R^2 is 0.9959.

$$\mu_X = \frac{\mu_m(C_S^0 + \alpha)}{K_C C_X + (C_S^0 + \alpha)} \tag{6}$$

where α was the residual carbon sources (expressed as glucose concentration equivalent) in the yeast extract or corn steep liquor (g/L).

In order to estimate Contois parameters, the linerized transformation of the Contois equation was used:

$$\frac{1}{\mu_X} = \frac{K_C}{\mu_m}\frac{C_X}{C_S^0 + a} + \frac{1}{\mu_m} \tag{7}$$

Based on the experimental data, firstly we can get $\alpha = 1.18$ because of its linearity. The maximal specific growth rate μ_m and the Contois constant K_C were determined from the slope of K_C/μ_m, and the intercept of $1/\mu_m$ by plotting the $1/\mu_X$ vs. $C_X/(C_S^0 + 1.18)$ on rectangular coordinates. The values of parameters were listed in Table 3. The value of R^2 was 0.9986, which indicated that the regression curve was in good accordance with the experimental data. The above results confirmed that the selected model was feasible for the kinetic simulation of cell growth (Fig. 3.).

In the batch culture process, the final cell concentration at a given glucose concentration can be expressed using the following equation [33]:

$$C_{X,max} = Y_{XS} C_S^0 + C_X^0 \tag{8}$$

where Y_{XS}, the ratio of the amount of cell formed to substrate glucose consumed, was the cell growth yield coefficient; $C_{X,max}$ was the maximum cell concentration (g/L); C_S^0 was the initial glucose concentration (g/L), and C_X^0 was the initial inoculation concentration (g/L). In this study, we inoculated spores in the culture, thus C_X^0 can be eliminated. Plotting C_S^0 vs. $C_{X,max}$, $Y_{XS} = 0.42061$ was obtained from the slope of the line.

Table 3
The kinetic parameters of *Rhizopus nigricans* TJ 108 growth and the bioconversion

Parameter	Value	Parameter	Value
μ_m (h^{-1})	0.2643	A	42.04
K_c (g/L)	0.2565	B	−35.12
a (g/L)	1.18	a	4.28
Y_{XS}	0.42061	b	2.44

3.3.2. Bioconversion kinetics study

According to the essay issued previously [10,34], only dissolved 16,17α-epoxyprogesterone can enter and influence the fungus growth and bioconversion process. But the concentration of the dissolved 16,17α-epoxyprogesterone is very low that the inhibition effect to enzyme can be negligible. Maxon et al. [11] reported that the steroid bioconversion of *R. nigricans* was a single-step enzymatic reaction. Considering the product inhibition existed in the bioconversion process due to the fact that bioconversion product were inside the cell, the following reaction mechanisms based on enzymatic reactions were proposed [35]:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (ES \rightleftharpoons EP) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$

By using pseudo-steady state approximation, one can easily derive the following equation.

$$k_1[E][S] + k_{-2}[E][P] = k_{-1}[ES] + k_2[ES] \quad (9)$$

$$[E_0] = [E] + [ES] \quad (10)$$

According to Eqs. (9) and (10), the following equation was obtained:

$$v = -\frac{d[S]}{dt} = \frac{k_1 v_1[S] - k_{-2} v_2[P]}{k_{-1} + k_2 + k_1[S] + k_{-2}[P]} \quad (11)$$

where $v_1 = k_2[E_0]$, $v_2 = k_{-1}[E_0]$, and [S] was dissolved substrate 16,17α-epoxyprogesterone concentration (g/L), [P] the dissolved product concentration (g/L), E_0 the initial enzyme concentration (units/g dcw).

The solubility of 16,17α-epoxyprogesterone is very low. We carried out the enzyme reaction in a heterogeneous reaction system in which most of the substrate is present in suspended particles. In this system, only dissolved substrate can be used by the enzyme due to the fact that the enzyme was inside the cell [10]. Here we introduced the following experiential equations:

$$\begin{aligned} [S] &= l_s S_t \\ [P] &= l_p P_t \end{aligned} \quad (12)$$

where S_t was the substrate 16,17α-epoxyprogesterone concentration at the time of t (g/L). P_t was the product concentration at the time of t (g/L). l_s and l_p were the ratios of dissolved substances in the whole 16,17α-epoxyprogesterone and the product, respectively. Furthermore, the molecular weights of substrate and product are very high and almost the same and the substrate does not constitute the cell components. When the byproduct was eliminated, we can obtain:

$$P_t = \frac{345}{328}(S_0 - S_t) \quad (13)$$

where S_0 was the initial substrate concentration (g/L). Integrating Eqs. (12) and (13) into Eq. (10):

$$v = -\frac{dS_t}{dt} = \frac{aS_t - b}{cS_t + d} \quad (14)$$

where

$$a = k_1 v_1 l_s + \frac{345}{328} k_{-2} v_2 l_p, \qquad c = (k_1 l_s^2 - \frac{345}{328} k_{-2} l_s l_s)$$
$$b = k_{-2} v_2 l_p S_0, \qquad d = k_{-1} l_s + k_2 l_s + \frac{345}{328} k_{-2} l_p l_s S_0$$

Hydroxyl enzyme is the inducing enzyme. In the initial stages of bioconversion, the product concentration was very low. After the inducing stage, the product concentration increased greatly. We regarded the end inducing stage as t_0. Integrating Eq. (14):

$$f^{-1}(S_t) = t = A \times \ln\left(\frac{aS_0 - b}{aS_t - b}\right) + B \times (S_0 - S_t) \quad (15)$$

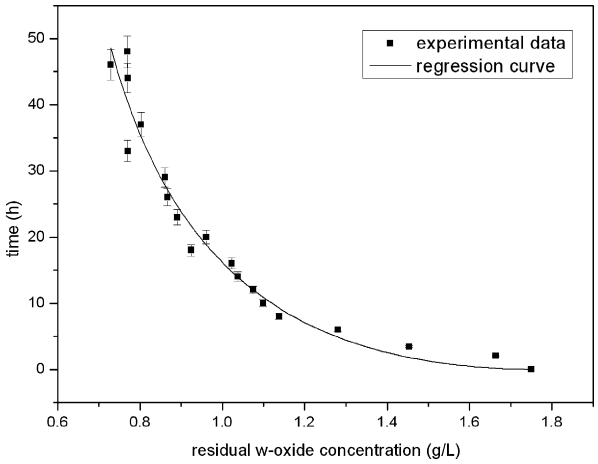

Fig. 4. Comparison between kinetic predictions and experimental bioconversion process at the substrate concentration of 1.75 g/L.

where $A = (1/a)((c/a)b + d)$, $B = (c/a)$, and t was the bioconversion time. Eq. (15) was a multiple linear function. Generally, the 16,17α-epoxyprogesterone substrate concentration added in the experiments was 1–2 g/L due to its toxicity, bioconversion rate and the following extract process. So we chose 1.75 g/L as the detailed study process. Values of the parameters $A = 42.04$; $B = -35.12$; $a = 4.28$; $b = 2.44$ were obtained using a multiple regression analysis of the software of Origin 7.0 based on the experimental data from the tests of bioconversion process. ($R^2 = 0.96$)

$$f^{-1}(S_t) = t = 42.04 \times \ln\left(\frac{7.49 - 2.44}{4.28S_t - 2.44}\right) - 35.12 \times (1.75 - S_t) \quad (16)$$

where t is the bioconversion time. Fig. 4 was the comparison between the prediction of the bioconversion kinetics and the experimentally determined bioconversion process at the substrate concentration of 1.75 g/L, from which it could be seen that the simulated values of bioconversion kinetics agreed well with the experiment data.

4. Conclusions

The optimization of bioconversion of 16,17α-epoxyprogesterone by *R. nigricans* TJ 108 was done by using uniform design: $X_1 = 25$ (g/L), $X_2 = 22$ (g/L), $X_3 = 0.1$ (g/L), $X_4 = 0.86$ (g/L), $X_5 = 6.55 \times 10^6$ spores/L and $X_6 = 3.4$. The model predicted bioconversion rate $y = 0.562$. The cell growth kinetics of *R. nigricans* TJ 108 was investigated at the initial glucose concentration from 0 to 30 (g/L), the temperature of 28 °C, and the initial pH of 3.4. The specific growth rate was proposed as $\mu = ((0.2643(C_S^0 + 1.18))/(0.2565C_X + (C_S^0 + 1.18)))$. The simulation of the bioconversion process at the substrate concentration of 1.75 (g/L) was: $f^{-1}(S_t) = t = 42.04 \times \ln((7.49 - 2.44)/(4.28S_t - 2.44)) - 35.12 \times (1.75 - S_t)$.

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