KINETIC ANALYSIS OF BATCH ETHANOL ACETYLATION IN ISOTHERMAL NON-STATIONARY MULTIPHASE SYSTEMS BY LYOPHILIZED MYCELIUM OF *ASPERGILLUS ORYZAE*

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ABSTRACT

A relatively complex network of reactions has been investigated, using as a network model the isothermal batch esterification of acetic acid with ethanol in *n*-heptane catalyzed by lyophilized mycelium of *Aspergillus oryzae*. The kinetic analysis was firstly carried out on the whole system, without any simplification, by means of the well-known integral method. Owing to the poor results obtained by this way, we developed an alternative approach, combining initial rates and integral analysis and reducing the number of empirical parameters to be determined by the use of equilibrium data. All the values of the parameters calculated according to this "composite" approach to kinetic analysis well correlate with experimental data.

Key words: *Aspergillus oryzae*, Complex network, Enzyme reactions, Esterification, Organic solvent

INTRODUCTION

The study of any reaction network in isothermal, non-steady-state systems could be carried out, in principle, by means of the integral method of kinetic analysis. As is known, such a method allows estimating, by regression, the parameters of a model using the experimental values of concentrations of the involved components along the time.

However, the results obtained by the integral approach are often scarcely useful for practical purposes, especially in the case of complex reaction networks and multiphase systems, when the correlation between model and experimental data is poor, or else when the values of some parameters are meaningless. Moreover, different values of these could fit equally well with experimental results, making it difficult to select the most reliable set of data.

Nevertheless, the effectiveness of the integral method can be greatly enhanced by reducing the number of parameters to be determined. This option doesn't necessarily require a simplification of the model, but it can also be done by direct calculation of some of the parameters by different methods and/or using chemical equilibrium data. These topics are discussed in the present work, taking as a reference model the esterification of acetic acid with ethanol in *n*-heptane, catalyzed by lyophilized mycelium of *Aspergillus oryzae*.

Various innovative processes of bioesterification in hydrophobic environment were extensively studied, making use of batch, isothermal reactors and different combinations of

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reagents, enzymes, organic solvents and operating conditions (2-4, 8-12), because most of them allow the enantiomeric resolution of racemic compounds of pharmaceutical significance, among which solketal (12), ketoprofen and ibuprofen (1).

Chemical transformations occurring in these systems are well described by the so-called "ping-pong" mechanism proposed by Yang and Russell (13), which is opposite to that presented by Jencks (7) for esters hydrolysis in water. Substitution of water with organic solvents, useful to move the chemical equilibrium towards the ester production, somewhat complicates some physical aspects of the kinetics, owing to the formation of different phases in the reacting fluid.

In previous works (2, 4), we studied the process kinetics applying the method of the initial rates to a simplified form of the model, so as to obtain results useful for practical applications. Some of these results are used here, implemented with equilibrium data, in order to simplify the kinetic analysis of the general, more comprehensive model, by means of the integral method.
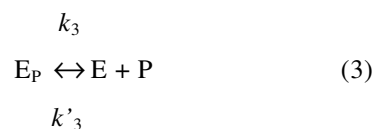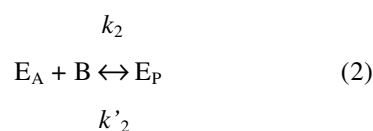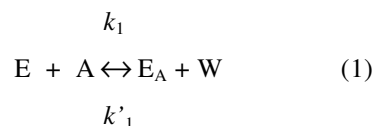
MATERIALS AND METHODS

Chemicals, mycelium preparation, procedures and analytical methods were previously described (2, 4, 10). The optimal process conditions were previously determined by an extensive experimental work, performed at different temperatures and concentrations of the lyophilized mycelium of *Aspergillus oryzae* MIM and substrates, and a simplified kinetic model based on the method of the initial rates was successfully used (2, 4). The kinetic investigation is extended in this work to the overall duration of the process, so as to evaluate the whole set of model parameters. All bioconversions were performed in triplicate at a temperature of 50 °C and using a lyophilized mycelium of *Aspergillus oryzae* MIM as a catalyst at concentrations of 10 and 20 g/L.

THEORY

Under particular circumstances, a rather complex network of reactions can be studied in a relatively simple way. The case of ethanol acetylation by mycelium-bound carboxylesterase of *Aspergillus oryzae* is chosen in this work as an example. The process was performed in organic solvent (*n*-heptane) (2, 4, 10) to shift the chemical equilibrium towards the esterification (6). The involved reaction network, corresponding to the so-called ping-pong mechanism (13), is:

$$E \ + \ A \xleftrightarrow[k'_1]{k_1} E_A + W \qquad (1)$$

$$E_A + B \xleftrightarrow[k'_2]{k_2} E_P \qquad (2)$$

$$E_P \xleftrightarrow[k'_3]{k_3} E + P \qquad (3)$$

where E is the active free enzyme, A the acetic acid, B the ethanol, P the ethyl acetate, E_A and E_P the complexes of the enzyme with acetic acid and ethyl acetate, respectively, W the water, while k_1, k_2, k_3 are the specific rate constants of the direct reactions (1-3) and k'_1, k'_2, k'_3 those of their respective inverse reactions.

Taking into accounts Equations (1-3), A, B and P balances can be written as:

$$dA/dt = - k_1 \, E \, A + k'_1 \, E_A \, W \qquad (4)$$
$$dB/dt = - k_2 \, E_A \, B + k'_2 \, E_P \qquad (5)$$
$$dP/dt = k_3 \, E_P - k'_3 \, E \, P \qquad (6)$$

the italic symbols of the chemical species representing their molar concentrations.

Because at the beginning of the process are $E_{A0} = E_{P0} = P_0 = 0$, the concentrations of the complexed and free enzyme at any instant are given by:

$$E_A = A_0 - A + B - B_0 \qquad (7)$$
$$E_P = B_o - B - P \qquad (8)$$
$$E = E_0 + A - A_0 + P \qquad (9)$$

Starting from the water balance in the phase where the reaction takes place:

$$W = W_0 + A_0 - A - K_r W \qquad (10)$$

one can write:

$$W = (W_0 + A_0 - A) / (1 + K_r) \qquad (11)$$

where $K_r W$ is the molar concentration of water in the non-reacting phase and K_r the corresponding partition coefficient.

Substitution of Equations (7-9) and (11) into Equations (4-6) gives:

$$dA/dt = - k_1 E_0 A - k_1 A (E - E_0) + k'_1{}^* W_0 E_A + k'_1{}^* E_A (A_0 - A) \qquad (12)$$
$$dB/dt = - k_2 B E_A + k'_2 E_P \qquad (13)$$

$$dP/dt = k_3 E_A - k'_3 E_P P \qquad (14)$$

where:

$$k'_1{}^* = k'_1 / (1 + K_r) \qquad (15)$$

RESULTS AND DISCUSSION

Figures 1 and 2 show the results of ethanol acetylation obtained under optimal operating conditions, i.e.: $T = 50°C$, concentration of the lyophilized mycelium, C_M, of 10 and 20 g/L, respectively. In fact, as previously stressed, under these conditions, the effect of enzyme inactivation can reasonably be neglected (2, 4).
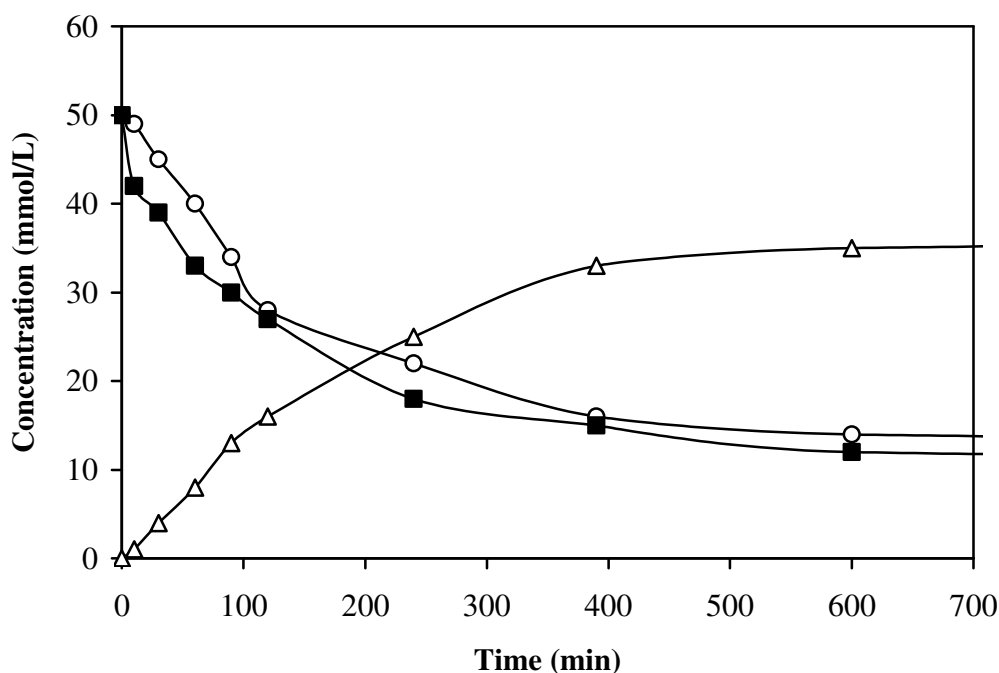

Figure 1. Experimental results of ethanol acetylation performed at 50°C with lyophilized mycelium of *A. oryzae* at concentration of 10 g/L. (O) Acetic acid; (■) ethanol; (△) ethylacetate. Starting conditions: acetic acid 50 mmol/L; ethanol 50 mmol/L. Equilibrium conditions were obtained after 2880 min as follows: acetic acid 9 mmol/L; ethanol 11 mmol/L; ethylacetate 38 mmol/L.
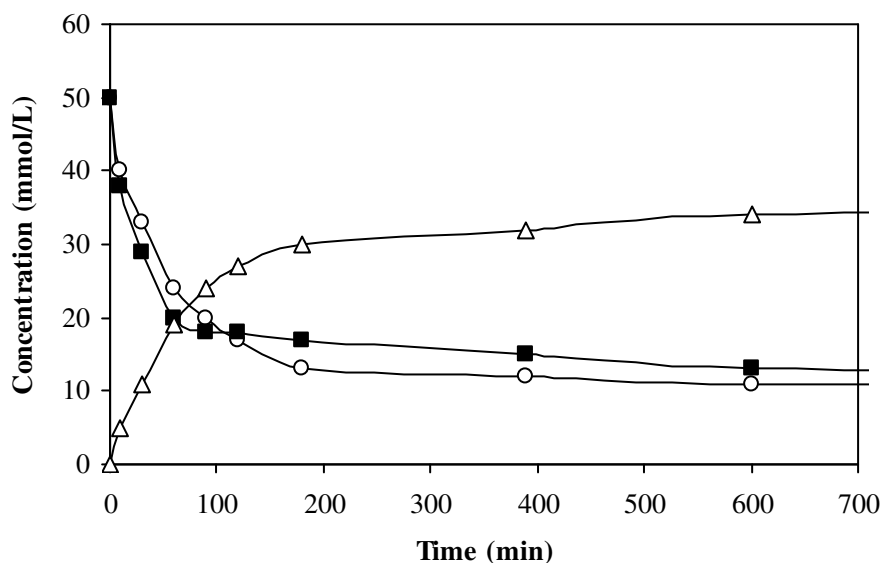
Figure 2. Experimental results of ethanol acetylation performed at 50°C with lyophilized mycelium of *A. oryzae* at concentration of 20 g/L. (O) Acetic acid; (■) ethanol; (△) ethylacetate. Starting conditions: acetic acid 50 mmol/L; ethanol 50 mmol/L. Equilibrium conditions were obtained after 2880 min as follows: acetic acid 8 mmol/L; ethanol 10 mmol/L; ethylacetate 37 mmol/L.

Integral method

As already remarked, the study of the process kinetics was firstly performed by means of the integral method (5), which requires formally integrating Equations (12-14) with respect to time:

$$A_t - A_0 = - k_1 E_0 \int_0^t A\,dt - k_1 \int_0^t A(E - E_0)dt + k'_1 * W_0 \int_0^t E_A \, dt +$$
$$+ k'_1 * \int_0^t E_A (A_0 - A)dt \qquad (16)$$

$$B_t - B_0 = - k_2 \int_0^t BE_A dt + k'_2 \int_0^t E_P \, dt \qquad (17)$$

$$P_t = k_3 \int_0^t E_P dt - k'_3 E_0 \int_0^t P dt - k'_3 \int_0^t P(E-E_0)dt \qquad (18)$$

then numerically calculating the integrals in Equations (16-18), taking into accounts Equations (7-9) and the experimental data, and finally estimating by linear regression the parameters appearing in Equations (16-18).

Unfortunately, the values of some parameters resulted to be meaningless (e.g., $k'_3 < 0$), probably due to the limitations of the method mentioned in the Introduction. Therefore, as discussed in the next session, a different approach is developed, presenting a two-step analysis as a tool for estimation of all relevant kinetic parameters.

Two-step method

The method adopted to calculate the kinetic parameters is based on the possibility of splitting the whole process into two stages. According to the experimental results, up to few tenths of minutes since the beginning of the esterification, the predominant reaction is certainly the attack of acetic acid to the active site of the enzyme, so the kinetics can be adequately described by Equations (4) and (9).

Making reference to the first 30 min of the process, where $P \cong 0$ and $W \cong W_0 \cong 0$, since the biocatalyst is lyophilized, combination of Equations (4) and (9) gives:

$$dA/dt = - k_1 A (E_0 + A - A_0) \qquad (19)$$

By integration, one can obtain:

$$A = A_o \frac{(E_o - A_o) \exp\left[- k_1(E_o - A_o)t\right]}{E_o - A_o \exp\left[- k_1(E_o - A_o)t\right]} \qquad (20)$$

From the experimental results obtained under the optimal conditions, we then estimated the values of the parameters k_1 and E_o listed in Table 1.

Table 1. Kinetic constant of acetylation and concentration of the active free enzyme.

C_M (g L^{-1})	k_1 (L mmol^{-1} h^{-1})	E_0 (mmol L^{-1})
10	0.161	11.4
20	0.133	23.3

As it can be seen, the values of k_1 are comparable one to the other, while those of the concentration of the active free enzyme are nearly proportional to the ones of the mycelium used in the runs. As regards the next phase of the process, the results demonstrate that equilibrium conditions (dA/dt = dB/dt = dP/dt = 0) were achieved after about 2 days, so:

$$A = A_\infty; B = B_\infty; P = P_\infty \tag{21}$$

$$E_A = E_{A\infty} = A_0 - A_\infty + B_\infty - B_0 \tag{22}$$

$$E_P = E_{P\infty} = B_0 - B_\infty - P_\infty \tag{23}$$

$$E = E_\infty = E_0 + A_\infty - A_0 + P_\infty \tag{24}$$

Then we obtain from Equations (12-14):

$$k'_1{}^* = k_1 A_\infty E_\infty / [(A_0 - A_\infty) E_{A\infty}] \tag{25}$$

$$k'_2 = k_2 B_\infty E_{A\infty} / E_{P\infty} \tag{26}$$

$$k'_3 = k_3 E_{P\infty} / (P_\infty E_\infty) \tag{27}$$

The value of $k'_1{}^*$ was directly calculated by Equation (25). As far as the other parameters are concerned, substitution of Equations (26-27) into Equations (17-18) gives:

$$B_t - B_0 = k_2 \int_0^t (B_\infty E_{A\infty} E_P / E_{P\infty} - B E_A) \, dt \tag{28}$$

$$P_t = k_3 \int_0^t [E_P - E_{P\infty} E P / (P_\infty E_\infty)] \, dt \tag{29}$$

The kinetic constants k_2 and k_3 were then calculated by means of the integral method, and, finally, k'_2 and k'_3 by Equations (26) and (27), respectively. Table 2 lists all the values estimated for these parameters.

The developed analysis allowed calculating in a simple and reliable way all the relevant kinetic parameters of the reaction network, including conventional rate constant and "pseudo kinetic" rate parameters, accounting for the active free enzyme concentration ($k'_1{}^*$ and k'_3).

Table 2. Kinetic parameters of the process steps following enzyme acetylation.

C_M (g L^{-1})	$k'_1{}^*$ (L mmol^{-1} h^{-1})	k_2 (L mmol^{-1} h^{-1})	k'_2 (h^{-1})	k_3 (h^{-1})	k'_3 (L mmol^{-1} h^{-1})
10	0.30	0.32	2.1	9.6	0.060
20	0.28	0.35	1.9	11.0	0.049

CONCLUSIONS

A two-step approach was proposed to analyze complex enzyme reaction networks in a multiphase system. Applicability of the method was demonstrated in the reaction scheme of bioesterification of acetic acid with ethanol in *n*-

heptane, catalyzed by lyophilized mycelium of *Aspergillus oryzae*. The first step of the framework considered as key reaction the attack of acetic acid to the active site of the enzyme; the subsequent step was based on the integral method, applied at the equilibrium conditions and allowed estimating all the kinetic parameters of the complete model. Such a novel approach could also be applied to other similar cases,

overcoming possible incongruities of the well-established integral method.

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