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Kinetics and modelling of an alcoholysis reaction catalyzed by cutinase immobilized on NaY zeolite

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Abstract

Fusarium solani pisi recombinant cutinase was immobilized by adsorption on NaY zeolite. The kinetics of the alcoholysis reaction of butyl acetate with hexanol in isooctane catalyzed by cutinase immobilized on NaY zeolite, was studied. The reaction kinetics is suggested to follow a Ping-Pong bi–bi mechanism in which competitive inhibition by excess of alcohols has been identified. No evidence of any significant external diffusional limitation has been detected. The time validation of the model was successfully achieved simultaneously for 15 experimental product evolutions in a batch stirred tank reactor (BSTR) for different initial reactant concentrations.

Keywords: Enzymatic catalysis; NaY zeolite; Cutinase; Enzyme immobilization; Alcoholysis

1. Introduction

Fusarium solani pisi recombinant cutinase immobilized by adsorption on NaY zeolite was used to catalyze the alcoholysis reaction of butyl acetate with hexanol to yield hexyl acetate and butanol, in isooctane.

In a previous study, cutinase was immobilized on several zeolites and its activity towards this alcoholysis reaction was studied [1]. The results obtained showed that zeolites can also be successfully used in this catalytic system, though the resulting catalytic properties are strongly dependent on the zeolite properties. One of the best activities was observed in preparations with NaY zeolite, which is commercially available. Due to these two reasons, NaY was the chosen zeolite for the kinetic study.

One of the most important parameter in this reaction is the water content. The influence of this parameter on the enzymatic activity was previously studied [1] and an optimum value was obtained; this value depends on the particular support used, but always corresponds to the same water activity ($a_w \approx 0.97$). This value was used in the following work as the optimum water activity value. The optimal immobilization conditions were also previously studied using a factorial design methodology [2].

The aim of this study was to investigate the kinetics of the alcoholysis of butyl acetate with hexanol catalyzed by the system mentioned above

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and to suggest a mechanism in order to determinate kinetic constants. This alcoholysis reaction belongs to the group-transfer reactions [3], and it is assumed to proceed through a reversible substituted-enzyme mechanism (Ping-Pong bi–bi mechanism).

2. Materials and methods

2.1. Enzyme and chemicals

F. solani pisi recombinant cutinase was produced by *Escherichia coli* WK-6 strain, a kind gift from Corvas International (Ghent, Belgium). The fermentation, extraction and purification were carried out following a protocol developed in our laboratory from the procedure of Lauwereys et al. [4]. 1-Hexanol and butyl acetate were purchased from Sigma. Isooctane was supplied by Riedel-de-Haën. All other chemicals including eluents and salts were of analytical reagent grade.

2.2. Preparation and characterisation of the supports

The sodium form of Y zeolite, NaY, was obtained from Union Carbide. The zeolite was characterized by X-ray diffraction in a model P Philips diffractometer equipped with a CuKα anticathode, in order to check crystallinity level. The dimensions of crystallites were evaluated by Scanning Electron Microscopy (SEM): values between 0.5 and 1 μm were obtained. This zeolite has pore apertures of 7.4 Å and a framework composition expressed as a Si:Al ratio of 2.7.

2.3. Enzyme immobilization

Cutinase was immobilized by deposition. Enzyme solution prepared in 150 mM sodium phosphate buffer (pH = 8.0) was added to the solid (25 mg of cutinase/g of support). This enzyme concentration was chosen after some preliminary studies [5]. The immobilization conditions (molarity and pH of enzyme solution) were chosen by factorial design methodology. After vortex-mixing for 1 min, the preparations were vacuum-dried overnight.

2.4. Water equilibration

The preparations were equilibrated in a closed vessel containing a saturated solution of potassium sulphate, giving a well-defined water activity (a_w = 0.97) at 30°C [6].

2.5. Protein and water determinations

The amount of immobilized enzyme remaining on the support, both before the reaction and after reaction and washing of the preparation with the organic solvent, was determined by a modified Folin assay [7]. The water content of the preparations was determined using a Mettler DL 18 Karl Fischer titrator [8].

2.6. Activity assay

The immobilized enzyme was used to catalyze the alcoholysis reaction of butyl acetate with hexanol in organic media (isooctane), resulting in the formation of hexyl acetate and butanol, at 30°C.

The reactions were carried out in a batch stirred reactor (BSTR), placed on an orbital stirrer operating at 400 rpm.

The hexyl acetate formation and butyl acetate consumption were followed by UV at 220 nm, using a HPLC system with a C18 reverse-phase column with isocratic elution and using a mixture of 60% acetonitrile and 40% water. The initial reaction rates were determined by linear regression.

3. Results and discussion

3.1. Mass transfer limitation study

Due to the use of an immobilized enzyme, the possible influence of external diffusional limitation

on the kinetics was investigated by carrying out several experiments at different rotating speeds (150–475 rpm). The values obtained for the initial rates increase slightly with rotation speed in the range from 150 to 300 rpm; above 300 rpm, no significant changes in the enzymatic activities were observed, which means that the external mass transfer limitations were not significant when the rotating speed was greater than 300 rpm. A rotation speed of 400 rpm was used in further experiments. The internal diffusion effect was not considered because the enzyme is only adsorbed on the external surface of the zeolite, as the zeolite pore apertures (7.4 Å) are too small to allow penetration of the enzyme.

3.2. Kinetic model

The alcoholysis reaction of butyl acetate with hexanol belongs to the group-transfer reactions [3]. We assume that the reaction proceeds through a substituted-enzyme mechanism (Ping-Pong bi–bi mechanism) and is reversible. In the first step, the active serine of cutinase (E) acts as a nucleophile to the carbonyl carbon of butyl acetate (AcBut), forming a stable tetrahedral intermediate (EAcBut). Then, butanol (But) is released and the structure reverts to the planar carbonyl flat plane acyl enzyme intermediate (EAc). In the last step, hexanol (Hex) also acts as a nucleophile, resulting to another tetrahedral intermediate, which restores the active site and produces the hexyl acetate (AcHex) [9]. Both alcohols react with the enzyme and form dead-end complexes, which are unable to participate in the reaction. The proposed reaction mechanism is illustrated in Fig. 1.

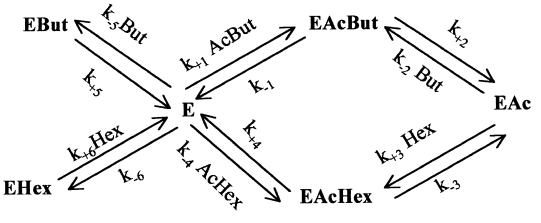

Fig. 1. Proposed reaction mechanism for the alcoholysis reaction of butyl acetate with hexanol.

The complete rate equation for substituted-enzyme mechanism with competitive inhibition of both alcohols is [3]:

$$v = \frac{\dfrac{V^{f}[\mathrm{AcBut}][\mathrm{Hex}]}{K_{i}^{\mathrm{AcBut}}K_{m}^{\mathrm{Hex}}} - \dfrac{V^{r}[\mathrm{AcHex}][\mathrm{But}]}{K_{i}^{\mathrm{But}}K_{m}^{\mathrm{AcHex}}}}{D} \quad (1)$$

$$D = \frac{[\mathrm{AcBut}]}{K_{i}^{\mathrm{AcBut}}} + \frac{K_{m}^{\mathrm{AcBut}}[\mathrm{Hex}]}{K_{i}^{\mathrm{AcBut}}K_{m}^{\mathrm{Hex}}}\left(1 + \frac{[\mathrm{Hex}]}{K_{si}^{\mathrm{Hex}}}\right) + \frac{[\mathrm{But}]}{K_{i}^{\mathrm{But}}}\left(1 + \frac{[\mathrm{But}]}{K_{si}^{\mathrm{But}}}\right) + \frac{K_{m}^{\mathrm{But}}[\mathrm{AcHex}]}{K_{i}^{\mathrm{But}}K_{m}^{\mathrm{AcHex}}} + \frac{[\mathrm{AcBut}][\mathrm{Hex}]}{K_{i}^{\mathrm{AcBut}}K_{m}^{\mathrm{Hex}}} + \frac{[\mathrm{AcBut}][\mathrm{But}]}{K_{i}^{\mathrm{AcBut}}K_{i}^{\mathrm{But}}} + \frac{K_{m}^{\mathrm{AcBut}}[\mathrm{Hex}][\mathrm{AcHex}]}{K_{i}^{\mathrm{AcBut}}K_{m}^{\mathrm{Hex}}K_{i}^{\mathrm{AcHex}}} + \frac{[\mathrm{AcHex}][\mathrm{But}]}{K_{i}^{\mathrm{But}}K_{m}^{\mathrm{AcHex}}}$$

The kinetic parameters are defined as:

$$V^{f} = \frac{k_{+2}k_{+4}}{k_{+2} + k_{+4}}$$

$$V^{r} = \frac{k_{-1}k_{-3}}{k_{-1} + k_{-3}}$$

$$K_{m}^{\mathrm{AcBut}} = \frac{(k_{-1} + k_{+2})k_{+4}}{k_{+1}(k_{+2} + k_{+4})}$$

$$K_{m}^{\mathrm{Hex}} = \frac{k_{+2}(k_{-3} + k_{+4})}{(k_{+2} + k_{+4})k_{+3}}$$

$$K_{m}^{\mathrm{But}} = \frac{(k_{-1} + k_{+2})k_{-3}}{(k_{-1} + k_{-3})k_{-2}}$$

$$K_{m}^{\mathrm{AcHex}} = \frac{k_{-1}(k_{-3} + k_{+4})}{(k_{-1} + k_{-3})k_{-4}}$$

$$K_{i}^{\mathrm{AcBut}} = \frac{k_{-1}}{k_{+1}}$$

$$K_{i}^{But} = \frac{k_{+2}}{k_{-2}}$$

$$K_{i}^{AcHex} = \frac{k_{+4}}{k_{-4}}$$

$$K_{si}^{But} = \frac{k_{+5}}{k_{-5}}$$

$$K_{si}^{Hex} = \frac{k_{+6}}{k_{-6}}$$

3.3. Initial velocity for the forward and reverse reactions

The concentration of hexanol was varied in the range of 20–2000 mM, maintaining constant the butyl acetate concentration at 2000 mM. The activity values are expressed in micromole of product formed per minute and per unit mass of enzyme (U/mg). Experimental observations (Fig. 2) clearly indicated that when the concentration of hexanol increased, the initial rate was proportionally increased, reaching a maximum at a critical concentration value. A subsequent increase in hexanol concentration led to a decrease in the initial rate. The hexanol acts as a competitive inhibitor above 1000 mM. Therefore, this was the chosen value to keep the hexanol concentration constant, varying the concentration of butyl acetate in the range of 100–2000 mM. Fig. 3 shows that no evidence of inhibition by butyl acetate was found. Initial rate studies were carried out under conditions in which the concentrations of both products can be neglected. Under these conditions, the general Eq. (1) can be simplified:

$$v = \frac{V^{f}[\text{AcBut}][\text{Hex}]}{K_{m}^{Hex}[\text{AcBut}] + K_{m}^{AcBut}[\text{Hex}]\left(1 + \frac{[\text{Hex}]}{K_{si}^{Hex}}\right) + [\text{AcBut}][\text{Hex}]} \quad (2)$$

Due to alcoholysis reversibility, the kinetic constants for the reaction between hexyl acetate and butanol were also determined. As in the previous procedure, the initial concentration of butanol was

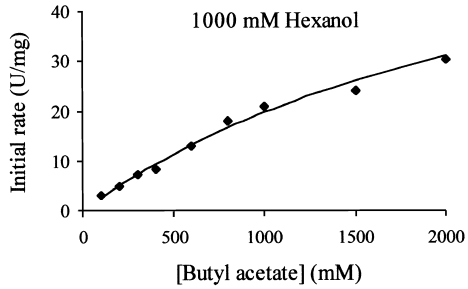

Fig. 3. Modelling of experimental initial rates for the forward reaction. The concentration of butyl acetate was varied, maintaining constant the hexanol concentration at 1000 mM. The symbols represent the experimental observations and the line is the model prediction.

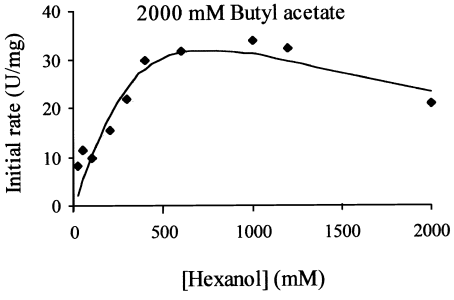

Fig. 2. Modelling of experimental initial rates for the forward reaction. The concentration of hexanol was varied, maintaining constant the butyl acetate concentration at 2000 mM. The symbols represent the experimental observations and the line is the model prediction.

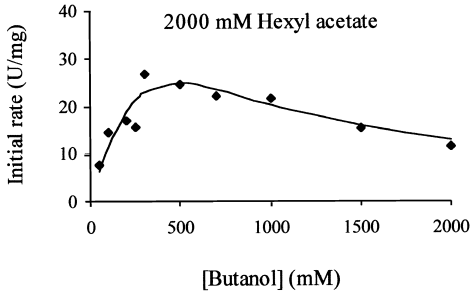

Fig. 4. Modelling of experimental initial rates for the reverse reaction. The concentration of butanol was varied, maintaining constant the hexyl acetate concentration at 2000 mM. The symbols represent the experimental observations and the line is the model prediction.

varied in the range of 50–2000 mM, maintaining constant the hexyl acetate concentration at 2000 mM. Experimental observations (Fig. 4) clearly indicate that butanol also acts as an inhibitor above 500 mM. Therefore, this was the chosen value to keep the butanol concentration constant, varying the concentration of hexyl acetate in the range of 100–2000 mM. Fig. 5 shows that no evidence of inhibition by hexyl acetate was found.

Initial rates studies were carried out under conditions in which the concentrations of both products can be neglected. Under these conditions, the general Eq. (1) for the reverse step can be simplified:

$$v = \frac{V^{r}[\text{AcHex}][\text{But}]}{K_{m}^{\text{But}}[\text{AcHex}] + K_{m}^{\text{AcHex}}[\text{But}]\left(1 + \dfrac{[\text{But}]}{K_{si}^{\text{But}}}\right) + [\text{AcHex}][\text{But}]} \quad (3)$$

It can be seen from Figs. 2–5 that the proposed model satisfactorily fitted the initial rate experimental data. The estimated kinetic constants are summarised in Table 1.

The same mechanism was used to model the same alcoholysis reaction promoted by cutinase encapsulated in AOT reversed micelles [10,11]. Internal diffusion limitations have been detected in this system as well as a competitive inhibition by the alcohols.

Table 1
Kinetic constants obtained for the direct and reverse reactions by fitting Eqs. (2) and (3) to the initial rates, and those obtained by fitting the numerically integrated complete rate Eq. (1) to the evolution of product amount

Forward reaction		Reverse reaction	
V^{f}	1.9×10^{2} U mg^{-1}	V^{r}	1.7×10^{2} U mg^{-1}
K_{m}^{Hex}	1.7×10^{3} mM	K_{m}^{But}	1.3×10^{3} mM
K_{m}^{AcBut}	1.0×10^{3} mM	K_{m}^{AcHex}	8.8×10^{2} mM
K_{si}^{Hex}	1.7×10^{2} mM	K_{si}^{But}	76 mM

Complete rate equation	
K_{i}^{AcBut}	54 mM
K_{i}^{But}	42 mM
K_{i}^{AcHex}	2.3×10^{4} mM

3.4. Comparison of model predictions and experimental observations for product evolution in a BSTR reactor

The complete rate Eq. (1) was numerically integrated and fitted to the experimental results of the product evolutions in the BSTR reactor, for several initial reactant concentrations. The kinetic parameters obtained in the initial rates study were used, and the other ones present in the rate equation (K_{i}^{AcBut}, K_{i}^{But} and K_{i}^{AcHex}) were estimated from these curve fittings (Table 1). The parameter K_{i}^{AcHex} is much higher when compared with the parameters K_{i}^{AcBut} and K_{i}^{But}.

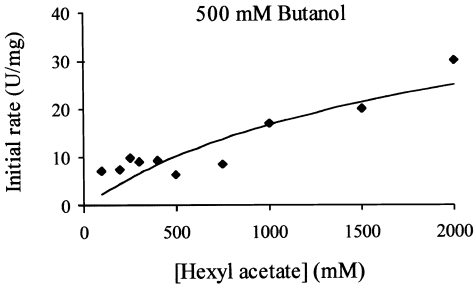

Fig. 5. Modelling of experimental initial rates for the reverse reaction. The concentration of hexyl acetate was varied, maintaining constant the butanol concentration at 500 mM. The symbols represent the experimental observations and the line is the model prediction.

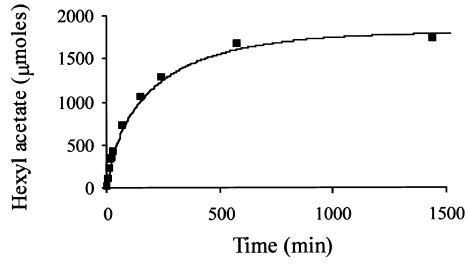

Fig. 6. Comparison between the experimental results for the evolution of the hexyl acetate (total number of moles in reaction volume) and model predictions. The initial substrates concentrations were 2000 mM butyl acetate and 600 mM hexanol. The symbols represent the experimental observations and the line is the model prediction.

The experimental data were successfully reproduced by the proposed model for the 15 curves obtained. As an example, one fitted curve and the experimental points are presented in Fig. 6; the good fitting quality is remarkable, which is similar for all the 15 BSTR runs.

The stability of the enzyme was tested and proven to be excellent in the reaction mixture in the range of substrate concentrations used.

4. Conclusions

A Ping-Pong bi–bi mechanism with competitive inhibition by the alcohols (substrate and product) was successfully proposed to model the alcoholysis reaction of butyl acetate with hexanol catalyzed by the immobilized cutinase on NaY zeolite. Both the initial rates, for the forward and reverse reactions, and the product evolutions in a BSTR reactor for 15 different initial concentrations are well reproduced by this model.

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