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Kinetics and modelling of transesterification reactions catalysed by cutinase in AOT reversed micelles

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Abstract

Kinetic studies were performed with cutinase to obtain the parameters V_{max}, K_M, k_{CAT} and K_S to the alcoholysis reaction of butyl acetate with hexanol and to the reversible reaction. The values of X_e and K_{eq} were also determined. Diffusional effects were detected for hexanol concentrations lower than 350 mM and the effectiveness factors determined. The integrated rate equation for a batch stirred tank reactor was applied considering a reversible kinetics and mass transfer effects. The experimental results were successfully described by the model for intermediate concentrations of hexanol (250–450 mM).

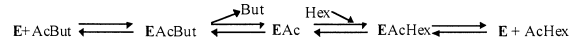
Keywords: Transesterification; Cutinase; Reversed micelles; Modelling; Kinetic constants

1. Introduction

Fusarium solani pisi recombinant cutinase, microencapsulated in bis(2-ethyl-1-hexyl) sodium sulfosuccinate (AOT)/isooctane reversed micelles, was used to catalyse the transesterification reaction of butyl cetate with hexanol to yield hexyl acetate and butanol. It is an alcoholysis that belongs to the group-transfer reactions. We assume that the reaction proceeds through a substituted-enzyme mechanism, (Ping-Pong bi–bi mechanism) and is reversible, which means the back reaction must be considered to define the equilibrium.

In a first step the active serine of cutinase (E) acts as a nucleophile to the carbonyl carbon of butyl acetate (AcBut), resulting a stable tetrahedral intermediate (EAcBut). At this point butanol (But) is released and the structure reverts to the planar carbonyl flat plane acyl enzyme intermediate (EAc).

At step 4, hexanol (Hex) acts as another nucleophile forming another tetrahedral intermediate which regenerates the active site and origins the product hexyl acetate (AcHex) [1].



Relevant parameters for the cutinase activity such as the temperature, buffer molarity, pH, the hydration degree, surfactant and substrates concentration were previously studied using the factorial design methodology [2].

Being AOT, a denaturing agent to cutinase the stability is a major point to optimise. The selected conditions included the presence of

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hexanol, a low Wo (water to surfactant molar ratio), a pH value of 8, close to cutinase isoelectric point, and a buffer molarity of 200 mM. Under these conditions the interactions between AOT and the protein are reduced and cutinase maintains a rigid structure. In the presence of 400 mM hexanol, cutinase incubated at 30°C in AOT reversed micelles (Wo = 2.7) remains 25 days without deactivation.

Besides avoiding cutinase unfolding, a low water content has the advantage of restricting the hypothesis of hydrolysis of the esters (substrate and product). In fact, it was not detected any acetic acid formation by GC analysis.

Kinetic studies were made in order to obtain the values of kinetic parameters that permit the development of a model to describe the experimental behaviour.

2. Materials and methods

2.1. Enzyme and chemicals

F. solani pisi cutinase was cloned and expressed in *Escherichia coli* WK-6 recombinant strain, a kind gift of Corvas International (Ghent, Belgium). Cutinase production and purification was carried out in our laboratory following the procedure of Lauwereys et al. [3].

1-Hexanol, butyl acetate and the surfactant bis(2-ethyl-1-hexyl) sodium sulfosuccinate, AOT were purchased from Sigma. AOT was used without further purification. Isooctane was supplied by Riedel-de-Haen. All other chemicals including eluents and salts were of analytical reagent grade.

2.2. Cutinase activity assays

Cutinase (0.3 mg ml^{-1}) was dissolved in 0.72% (v/v_{total}) 200 mM phosphate buffer at pH 8 and was further microencapsulated in 150 mM AOT in isooctane (Wo = 2.7) by the injection method. The reactions were performed in a batch stirred tank reactor (BSTR) with an agitation of 600 rpm at 30°C and followed for 24 h.

The concentrations mentioned are all referred to the total volume (5 ml) except the buffer molarity which is considered only in the aqueous phase. The pH values mentioned are those of the buffer in which cutinase is dissolved to prepare the aqueous phase.

The initial velocity measurements were made following the hexyl acetate formation by U.V. at 220 nm using a HPLC system with a C_{18} reverse-phase column and isocratic elution.

GC analysis was the selected method to determine the final concentrations of all reactants and products in order to calculate the value of the equilibrium constant (K_{eq}). The samples were analysed in a 5890 Hewlett Packard series II Gas Chromatograph, using an HP-5 capillary column (crosslinked 5% PH ME siloxane), a FID detector and nitrogen as a carrier gas.

2.3. Modelling method

The model used to fit the results was built with a Excel 7.0 workbook from Microsoft® and was applied to the experimental results with the solver of the same program in a Windows '95 environment.

3. Results and discussion

The initial velocity measurements were made in the absence of products and the concentration of hexanol was varied in the range 100–1000 mM maintaining constant butyl acetate at 1 M. This procedure leads to a Michaelis–Menten equation with respect to the hexanol [4].

The analysis of the results revealed the existence of diffusional effects for alcohol concentrations lower than 350 mM. Due to alcoholysis reversibility, the kinetic constants for the reaction between hexyl acetate (1 M) and butanol (variable) were also determined.

The experimental results were adjusted by applying the integrated rate equation for BSTR considering a Michaelis–Menten reversible kinetics. The effectiveness factor was related to hexanol concentration. To obtain the parame-

Table 1
Kinetic constants obtained for the reversible alcoholysis reaction catalysed by cutinase

	Butyl acetate + hexanol → hexyl acetate + butanol	Hexyl acetate + butanol → butyl acetate + hexanol
V_{max}	21.5 mM min^{-1}	17.6 mM min^{-1}
K_M	214 mM	292 mM
k_{CAT}	1.577×10^3 min^{-1}	1.291×10^3 min^{-1}
K_S	7.4 mM^{-1} min^{-1}	4.4 mM^{-1} min^{-1}

ters, maximum velocity or limiting rate (V_{max}) and the Michaelis constant(K_M), a graphical representation of the Michaelis–Menten equation of s/v against s (Hanes plot) was used. k_{CAT} refers to the catalytic constant and defines the number of catalytic processes that a molecule of enzyme can catalyse in a unit time. The specificity constant, K_S defines the ability of the enzyme to distinguish between different substrates [4,5].

From the aforementioned and observing the values of K_S in Table 1 it is possible to conclude that cutinase has more affinity to hexanol when compared with butanol.

The large values of K_M obtained could be related to the partitioning of hexanol between the isooctane and aqueous microenvironment where the reaction takes place. The diffusional effects observed for low concentrations of hexanol and the fact that it interposes in the surfactant layer acting as a co-surfactant reinforce this hypothesis [6].

The value of K_{eq} was experimentally determined to be 1.23 which is the average of seven different experiments with a standard deviation of 0.0884.

Mass transfer experiments were performed to determine the effectiveness factor as a function of hexanol concentration and the results are plotted in Fig. 1.

From the results referred above and considering the equation for a Michaelis–Menten reversible reaction, we have

$$V = \frac{V_S K_P S - V_P K_M P}{K_M K_P + K_P S + K_M P}$$

Integrating and assuming $V_{max} = V_S = V_P$, the following equation can be obtained:

$$\eta V_{max} t = S_o X_e \left\{ X\left(1 - \frac{K_M}{K_P}\right) + \left(\frac{K_M}{S_o} + 1 - X_e + X_e \frac{K_M}{K_P}\right) \times \ln\left(\frac{X_e}{X_e - X}\right) \right\}$$

The conversion degree at equilibrium (X_e) reaches 73% for concentrations of hexanol in

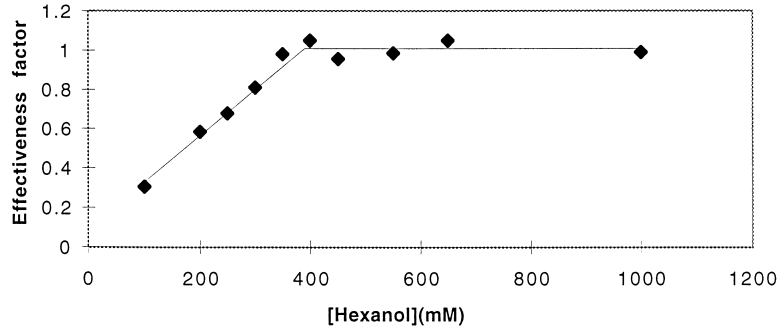

Fig. 1. Dependence of the effectiveness factor on the concentration of hexanol. The values are based on the comparison of initial velocities experimentally measured and those expected by Michaelis–Menten reversible kinetics.

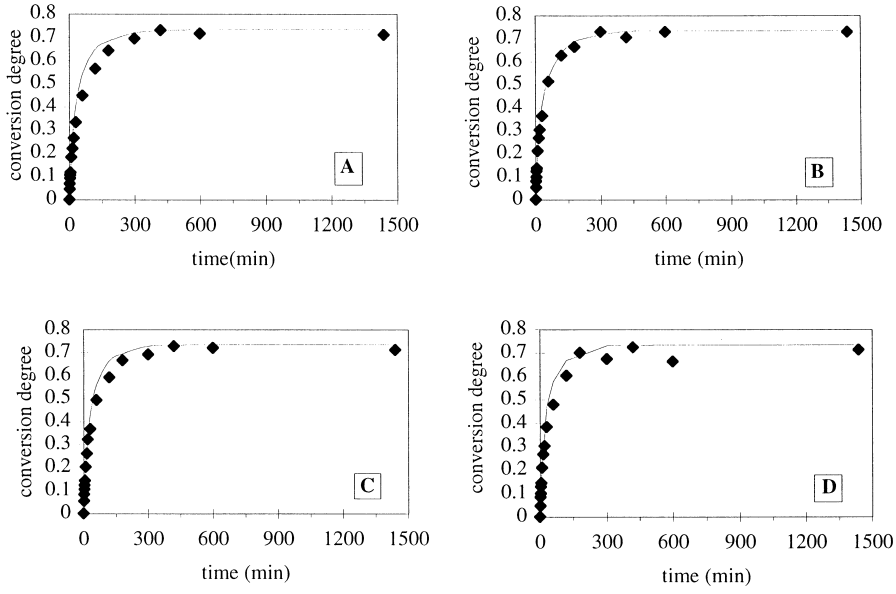

Fig. 2. Modelling of the experimental results obtained using a fixed concentration of butyl acetate and 250 mM (A), 300 mM (B), 350 mM (C), and 400 mM (D) of hexanol. The symbols represent the experimental observations and the lines are the model predictions.

the range 100–350 using 1 M butyl acetate. Above 350 mM of hexanol there is a gradual shift in equilibrium and the final conversion decreases until 50%, when using equimolar concentrations of both substrates.

The kinetic and mass transfer results were used to model the performance of a batch stirred tank reactor (BSTR). The experimental results were adjusted by applying the integrated rate equation for BSTR considering a Michaelis–Menten reversible kinetics associated with the effectiveness factor (Fig. 2).

Despite the good correlations obtained with intermediate concentrations of hexanol, there is some deviation when the extreme concentrations of 100 mM and 1000 mM are approached. A possible explanation is that a concentration of 100 mM hexanol is not enough to retain cutinase full activity and so the deviation is due to a deactivation process which is not taken into account by the model.

A concentration of hexanol higher than 650 mM leads to higher conversion rates of substrates into hexyl acetate and butanol and there is the possibility of these products to act as competitive inhibitors to the active site of the enzyme.

4. Conclusions

The development of the present model is a first approach to a more general interpretation of the alcoholysis reaction catalysed by cutinase from *F. solani pisi*. The kinetic parameters of V_{max} and K_M were calculated for the direct reaction as well as for the reversible reaction. Moreover, the values of X_e and K_{eq} were experimentally determined. A model was developed with the mentioned constants and additionally the effectiveness factor values were included.

The application of the model to the experimental data gives a good correlation in the range of 250–400 mM hexanol. The inhibition of butanol exists but it was not yet considered in the model although it is predictable that its inclusion will improve the fitting as it will be

helpful to describe the whole reaction equilibrium process.

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