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Analysis of a BSTR reactor for triglyceride hydrolysis with an immobilised cutinase

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Abstract

Fusarium solani pisi recombinant cutinase was immobilised by adsorption on NaY zeolite. A BSTR reactor was developed for the utilisation of the immobilised enzyme on the hydrolysis of triglycerides in non-conventional media. For a reaction medium with 4% of water and 96% of organic phase, high conversions were obtained. The kinetic parameters of the immobilised enzyme were determined: $V_{max} = 35.8$ U/g and $K_M = 186$ mM. External mass transfer limitations were observed with linear velocities lower than 85 cm/s, and the effectiveness factors were determined. For triglyceride concentrations between 20 and 250 mM a first-order kinetics was observed, due to diffusional control the substrate concentration at the support surface (S_S) was always lower than K_M. For linear velocities between 29 and 107 cm/s, a first-order kinetics was also observed when lower agitation rates were used—diffusional control—and loss of enzymatic activity occurred for linear velocities higher than 85 cm/s. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Cutinase; NaY zeolite; Immobilisation; BSTR reactor; Triglyceride hydrolysis; Kinetic studies

1. Introduction

Cutinase from *Fusarium solani pisi* is a lipolytic enzyme that catalyses the hydrolysis of the water-insoluble biopolyester cutin which covers plant surfaces. This enzyme is also able to hydrolyse soluble esters and triglycerides of a wide variety of chain-lengths as efficiently as esterases and lipases do [1]. The enzyme active site is composed by the usual catalytic triad of lipases (Ser–Asp–His) and is located in a crevice between two hydrophobic loops (residues 80–90 and residues 182–189). These loops seem to be involved in both interfacial and substrate binding [2]. Its catalytic versatility makes cutinase an interesting enzyme to be applied to biotechnological processes such as hydrolysis of milk fat, production of ω3-polyunsaturated fatty acid, manufacture of household detergents, production of optically pure compounds, production of emulsifiers and oil waste treatment [3,4].

Several methodologies are known for the use of lipases in organic solvents and one practical approach is to immobilise the enzyme on a solid support. The immobilisation often increases the thermostability of the enzyme, the dispersibility in the organic solvent is improved and the immobilised enzyme can be reused and continuously operated in a reactor. BSTRs are com-

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monly used and are extremely versatile reactors and easy to operate [3].

In the present study, a *F. solani pisi* recombinant cutinase was immobilised by adsorption on a hydrophilic support—NaY zeolite. A BSTR reactor was developed for the utilisation of the immobilised enzyme on the hydrolysis of triglycerides in non-conventional media. The kinetic characterisation of the immobilised enzyme was carried out, taking into account the effect of triglyceride concentration and the effect of external mass transfer limitations.

2. Materials and methods

2.1. Chemicals

Escherichia coli containing the gene of *F. solani pisi* recombinant cutinase was a gift of Corvas International (Ghent, Belgium). Cutinase production and purification was done according to previously published methods [1]. NaY zeolite was obtained from Union Carbide, (Danbury, USA). Tricaprylin (> 95% purity) was purchased from Fluka, (Buchs, Switzerland). Caprylic acid (99% purity) was from Sigma, (St. Louis, MO, USA). Karl Fischer reagent was from Riedel de Haën, (Seelze, Germany). All salts were purchased from Merck and iso-octane was obtained from Riedel de Haën.

2.2. Enzyme immobilisation

Cutinase was immobilised by deposition onto zeolite support. An enzyme solution (10 mg/ml), prepared in 50 mM sodium carbonate buffer (pH 9.0) was added to the solid support (25 mg of cutinase per gram of zeolite). After vortex mixing for 3 min, the preparation was vacuum dried for at least 48 h.

2.3. Protein and water determinations

The protein content of the immobilised enzyme was determined by a modified Folin assay [5], using BSA standards. Water content of the immobilised cutinase was determined using a Mettler DL18 Karl Fischer titrator.

2.4. Activity assay

The enzymatic activity was determined by the hydrolysis of tricaprylin in iso-octane (2,2,4-trimethylpentane), following the formation of caprylic acid by the method of Lowry and Tinsley [6]. One unit (U) of enzymatic activity is defined as 1 μmol of fatty acid formed per minute.

The enzyme preparations (500 mg) were hydrated, by the addition of a controlled amount of water, followed by the addition of a solution of tricaprylin in organic solvent to start the reaction. A reaction volume of 50 ml was used, with a water/organic solvent ratio of 4/96 (v/v). The reactions were carried out in a BSTR reactor, stirred with a magnetic bar, at 30°C and at the immobilisation pH.

3. Results and discussion

3.1. Characterisation of the immobilised cutinase

In systems containing enzymes immobilised on solid supports and working in organic media, the support has a significant influence on the total enzymatic activity and can also displace the reaction equilibrium (hydrolysis toward synthesis). Zeolites are crystalline aluminosilicates, with a structure consisting on an infinite three-dimensional structure of SiO_4 and AlO_4^- tetrahedra molecules linked to each other by shared oxygen. The properties of a particular zeolite depend mainly on its structure and usually, they can be modified by thermal and chemical treatments, which gives the possibility of regulating properties as acidity, hydrophobicity and selective adsorption. The NaY zeolite presents a hydrophilic character, with a remarkable water affinity and a high adsorption capacity [7], which

makes this material a good support for cutinase immobilisation and utilisation on hydrolytic reactions.

A BSTR reactor was developed for the utilisation of the immobilised enzyme on the hydrolysis of tricaprylin in non-conventional media, using a ratio of aqueous:organic phases of 4:96 (v/v). The immobilised enzyme activity, productivity and final conversion were evaluated using several agitation rates and the results are presented in Table 1. The enzyme activity and productivity increase with the agitation rate up to a linear velocity of 85 cm/s, and after that, a decrease was observed. These results indicate the existence of mass transfer limitations, essentially at the lowest agitation rates, and also indicate that strong agitation rates have a negative effect on the enzyme activity and productivity, probably because the enzyme becomes deactivated by the strong shear forces formed.

A linear velocity of 58 cm/s was chosen to determine the apparent kinetic parameters of the immobilised cutinase. The enzyme presented, a Michelis–Menten kinetics and the apparent kinetic parameters were calculated using a non-linear regression of the Michelis–Menten equation. The values obtained were 35.8 ± 4.4 U/g for the $V_{\text{max app}}$ and 186 ± 39 mM for the $K_{\text{M app}}$.

The adsorption of cutinase occurred only on the external surface of the NaY zeolite because the pore size of the support is too small (7.5 Å) in relation to the enzyme dimensions ($30 \times 30 \times 45$ Å). Thus, only external mass transfer limitations may occur. The external effectiveness factors (η) were determined by the graphical representation of the dependence of η on the dimensionless substrate concentration, β ($\beta = S/K_M$) and the dimensionless modulus, μ ($\mu = V_{max}/K_M \times k_L a$) [8].

Table 2
First-order rate constants (k) for the hydrolysis of tricaprylin with the immobilised cutinase, using different substrate concentration

$[\text{Tricaprylin}]_{\text{BULK}}$ (mM)	$[\text{Tricaprylin}]_{\text{SURFACE}}$ (mM)	k (hr^{-1})
20	4.15	6.32×10^{-2}
50	10.5	3.97×10^{-2}
100	22.0	2.59×10^{-2}
150	34.3	2.46×10^{-2}
200	48.4	2.46×10^{-2}
250	63.4	3.06×10^{-2}

The enzyme activity was determined at 58 cm/s, 30°C and pH 9.0 (immobilisation pH). A ratio of aqueous:organic phases of 4:96 (v/v) was used.

3.2. Effect of the substrate concentration on the kinetics of the immobilised cutinase

The kinetic behaviour of the immobilised enzyme was investigated using several tricaprylin concentrations. The hydrolytic reactions were performed at a linear velocity of 58 cm/s, 30°C and pH 9.0 (immobilisation pH). A ratio of aqueous: organic phases of 4:96 (v/v) was used. A first-order kinetics was obtained due to diffusional control as the substrate concentration at support surface was always much lower than $K_{\text{M app}}$. The first-order rate constants

Table 1
Effect of the linear velocity on the enzyme activity and productivity of immobilised cutinase and final conversion

Linear velocity (cm/s)	Activity (U/g of zeolite)	Productivity (U/g of zeolite)	Conversion (%)	Coupling yield (%)
29	2.11	1.96	100	46
34	5.31	2.48	97	47
40	10.1	3.09	87	46
58	12.8	6.59	88	41
71	19.5	15.2	95	40
85	42.3	7.21	88	41
107	36.8	4.56	90	43

The enzyme activity was determined by the hydrolysis of tricaprylin (100 mM) in iso-octane, 30°C and pH 9.0 (immobilisation pH). A ratio of aqueous:organic phases of 4:96 (v/v) was used.

Table 3
Effect of the agitation rate on mass transfer and on the first-order rate constants (k) for the immobilised cutinase

Linear velocity (cm/s)	$[\text{Tricaprylin}]_{\text{SURFACE}}$ (mM)	$k_L a$ (min^{-1})	μ	k (h^{-1})
29	3.51	6.88×10^{-5}	28.0	1.02×10^{-2}
34	9.14	1.85×10^{-4}	10.4	1.21×10^{-2}
40	18.2	3.90×10^{-4}	4.94	1.75×10^{-2}
58	22.0	4.86×10^{-4}	3.96	2.59×10^{-2}
71	38.7	1.01×10^{-3}	1.91	6.29×10^{-2}
85	100	–	–	2.47×10^{-2}
107	100	–	–	2.11×10^{-2}

The enzyme activity was determined by the hydrolysis of tricaprylin (100 mM) in iso-octane, 30°C and pH 9.0 (immobilisation pH). A ratio of aqueous:organic phases of 4:96 (v/v) was used.

were determined and are presented in Table 2, with the calculated substrate concentration at the catalytic surface and the substrate concentration in the bulk solution.

3.3. Effect of the agitation rate on the kinetics of the immobilised cutinase

The kinetic behaviour of the immobilised enzyme was investigated using several linear velocities. The hydrolytic reactions were performed at a tricaprylin concentration of 100 mM, 30°C and pH 9.0 (immobilisation pH). A ratio of aqueous:organic phases of 4:96 (v/v) was used. For the linear velocities evaluated, a first-order kinetics was also observed. However, when the agitation rate increased (≥ 40 cm/s) the experimental values can also be represented by a Michaelis–Menten kinetics. For linear velocities higher than 85 cm/s, loss of enzyme activity was observed and only the first-order kinetic model can fit the experimental values. The substrate concentration at the catalytic surface, the mass transport coefficient ($k_L a$), the dimensionless modulus (μ) and the first-order rate constants were determined and are presented in Table 3.

4. Conclusions

A BSTR reactor was developed for the utilisation of the immobilised cutinase on the hydrolysis of triglycerides in non-conventional media. For a reaction medium with 4% of water and 96% of organic phase, high conversions were always obtained. A first-order kinetics was observed with different substrate concentrations due to diffusional control and loss of enzyme activity occurred for the higher agitation rates.

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