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Kinetic model for the esterification of ethyl caproate for reaction optimization

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ABSTRACT

The present work aims to achieve additional insight on a mechanism describing the fundamental steps involved in the esterification reactions catalyzed by cutinase. The synthesis of ethyl caproate has been used as a model system to obtain a suitable kinetic model to estimate the activation energies involved in the various steps of the reaction pathway.

Kinetic measurements have been made for the enzymatic esterification of caproic acid with ethyl alcohol catalyzed by recombinant *Fusarium solani pisi* cutinase expressed in *Saccharomyces cerevisiae* SU50. Different temperature conditions, from 25 to 50 °C, were tested for two different alcohol/acid molar ratios ($R = 1$ and $R = 2$). The third ordered Ping Pong Bi Bi mechanism with alcohol inhibition was shown to be able to describe the experimental results. The model shows that the productivity decreases as the reaction temperature increases.

1. Introduction

Alkyl esters, an important part of aroma compounds, are commonly used in food, cosmetic and pharmaceutical industries. The use of biocatalysis, using hydrolytic enzymes, for the synthesis of aroma compounds of biological interest has gained a particular interest during the last decades. Advantages of using the enzymatic synthesis in non-aqueous media significantly expand the possibilities for industrial applications [1–3].

There are several reasons for the use of enzymes, mainly lipases and esterases, for the synthesis of short chain acid esters. Enzyme esterification is an interesting option when compared to chemical synthesis as it has the advantages of being able to be carried-out under mild reaction conditions and to ensure the high quality and purity of the products; also have been considered as natural components by food regulatory agencies [4,5].

Fusarium solani pisi (*F.s.pisi*) cutinase activity in hydrolysis, esterification and transesterification has been extensively exploited in recent years and several applications in different industrial fields have been proposed [6]. Numerous report of using cutinases in different reaction media, often dissolved in aqueous solution but also suspended as a powder or immobilized, have been reported [7–18]. Fundamental studies on the hydrolysis of triglycerides [7] clarification of its mechanism regarding stereo-selectivity and specificity [8], and studies of esterification reactions [9–18] were performed with lyophilized cutinase.

The sub-family of cutinases consists of about 20 members, based on amino-acid sequence similarity, which display hydrolytic activity on cutin polymers and efficiently hydrolyze soluble small carboxylic esters and emulsified triacylglycerols.

Cutinase belongs to the family of serine hydrolases containing its catalytic serine centre at the middle of a sharp turn between a β-strand and a α-helix [6]. The catalytic triad, *Ser-120*, *Asp-175* and *His-188*, is accessible to the solvent and can accommodate different substrates. The esterification reaction was shown to follow a Ping-Pong Bi Bi mechanism [15,16]. The serine in the active centre of the enzyme is a very strong nucleophile, which attacks the carbonyl group of the acid (Ac), forming a stable tetrahedral intermediate acyl enzyme complex. The acyl enzyme complex is stabilized by the oxyanion hole. Water is then released and the structure reverts to the planar carbonyl flat plane acyl enzyme intermediate (EA_c). The alcohol (Al) acts afterwards as a new nucleophile and links to the tetrahedral intermediate. Subsequently, as the final step, the resolution of tetrahedral complex yields the ester (Es) and the free enzyme (E).

The industrial application of any chemical reaction requires the knowledge of its kinetics, in particular by using a suitable kinetic model, to allow the description of a chemical reaction and its

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Ac	acid
Al	alcohol
Es	ester
E	free enzyme
$[E]_t$	total enzyme concentration, mM
EA_c	enzyme acyl complex
EA_{c2}	enzyme acyl complex with two bound acid molecules
EA_{c3}	enzyme–acyl complex with three bound acid molecules
EA_l	enzyme–alcohol complex
THC	tetrahedral intermediate acyl enzyme complex
$K_{a1}, K_{a2}, K_{a3}, K_{b1}, K_{b1}, K_{b1}$	equilibrium constants
K_i	inhibition coordination constant
$k_{a1}, k_{-a1}, k_{a2}, k_{-a2}, k_{a3}, k_{-a3}, k_{b1}, k_{-b1}, k_{b1}, k_{-b2}, k_{b3}, k_{-b3}$	rate constants, $mM^{-1}\,min^{-1}$

optimization over an extensive range of reagents composition and presence of inhibitors and concentrations, and reaction conditions such as temperature, pH, pressure, etc.

In order to ensure that the model is applicable over a wide range of experimental conditions it should be preferentially based on a mechanistic scheme describing the fundamental steps involved in the reaction. The development of this kind of models can also be used to provide insight into the processes that are taking place.

From previous studies [12,15] *F.s. pisi* cutinase expressed in *Saccharomyces cerevisiae* showed a significant potential for the synthesis of short chain ethyl esters in isooctane. The kinetic parameters of this system in terms of Ping Pong Bi Bi models were estimated, under isothermal conditions, to improve the description of the reaction system. However, the impact of different temperature regimes in the reaction progress is very important and requires additional study. The impact of temperature in the ester yield and rate is difficult to predict because it may affect reaction efficiency in conflicting ways and this knowledge is required for system optimization. On one hand a temperature raise will have a positive effect on the reaction rates, as expected from the transition state theory. On the other hand, higher temperatures may disrupt the enzyme's tertiary structure, causing it to lose its catalytic activity.

Therefore, the aim of this work was evaluation of activation energy of the different steps of the cutinase catalyzed esterification to analyze temperature impact in the production of ethyl caproate.

2. Materials and methods

2.1. Enzyme and chemicals

Caproic acid (C_6) (99.0%, Fluka, Germany) and ethanol abs. (VWR, Germany) were used for ester synthesis, while iso-octane (99.5%, Fluka, Germany) was used as organic solvent and *n*-decane (VWR, Germany) was used as an internal standard for gas chromatography (GC). Sodium sulfate anhydride (Acros, Geel, Belgium) was used to dry iso-octane as organic media of esterification reactions. Saturated salt solution of sodium chloride (Panreac, Spain) was used to control water activities of enzyme and substrates. All other chemicals used were of analytical grade.

Fusarium solani pisi cutinase wild-type was biosynthesized by recombinant *S. cerevisiae* SU50 strain as described by Calado et al. [19]. The isolation and purification and characterization of cutinase excreted by recombinant *S. cerevisiae* SU50 strain was carried out by according to previous published protocols [12,15]. Lyophilized pure cutinase was stored at −20 °C before used in esterification reactions. Activities of lyophilized cutinase preparations were of $240 \pm 10\,U\,m^{-1}$ of reaction medium according the p-NPB method [11].

Table 1
Experimental conditions for the set of experiments used in the fitting of the different models.

T, °C	Al/Ac = 1		Al/Ac = 2	
	Ethanol, mM	Caproic acid, mM	Ethanol, mM	Caproic acid, mM
25	208	205	217	121
30	231	214	210	117
35	211	199	210	110
40	216	219	204	114
45	214	213	203	119
50	213	197	210	117

All concentrations are given in mM. Enzyme concentration is equal to 0.1 mM in all runs and the specific activity was $240 \pm 10\,U\,ml^{-1}$ of reaction media.

2.2. Enzymatic esterification

The esterification of acid and alcohol by cutinase was carried out in iso-octane as organic solvent, as previously explained [15]. The enzymatic ester synthesis was performed in an incubator (AGITORB 16OE, Aralab, Portugal) at various temperatures (25–50 °C), and cutinase concentration of 2 mg/ml of reaction mixture. Experiments were performed at least in duplicate and the experimental error was estimated less than 8%. Samples were withdrawn periodically using a needle, without destroying the rubber cap, and analyzed by GC. The reaction yield was calculated according to the molar ratio between the ethyl ester and respective limiting substrate, in this case acid.

2.3. Methods for monitoring substrate and ester concentrations

The concentrations of ethanol, caproic acid and ethyl caproate were determined using a Hewlett–Packard model 5890 gas chromatograph, equipped with a flame ionization detector (FID). A WCOT Fused Silica coating CP Chirasil-Dex CB column, 25 m × 0.25 mm, DF = 0.25 (Varian Inc.) was used to separate the components in the reaction mixture. *n*-Decane was used as an internal standard in the quantification of ethyl esters and respective substrates concentrations in the reaction media. Nitrogen was used as carrier gas. The oven temperature was held at 50 °C for 4 min before being raised to 160 °C for 1.67 min at 15 °C min^{-1}; the injector temperature was set at 200 °C and the detector temperature was set at 250 °C.

2.4. Kinetic model

The base model development was described in a previous work [15] where it was applied to data at a single temperature. To implement the different models the appropriate differential and equilibrium equations were written for each kinetic model and an approximate global reaction rate was obtained using the quasi-steady-state approximation. This rate equation was used to compute the time-course evolution of the different species involved by numerical integration of the material balances using the Euler method.

The model was fitted to the experimental data (all the available experiments were used simultaneously) by a least squares procedure where the objective function consisted of the sum of the square of relative errors of the ethanol, caproic acid and ethyl caproate concentrations measured in all experiments. The experimental conditions, for the set of experiments used in fitting procedure are given in Table 1. This procedure was carried-out using a Microsoft Excel 2003 spreadsheet and the estimation of the