Kinetics of Ethyl Acetate Formation by Lipase in Organic Solvent and Solvent-Free System

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Manufacturing of ethyl acetate by lipase in both organic solvent and solvent-free system was studied and the apparent kinetics of the reaction was analyzed following the Michaelis—Menten model. It was found that substrate (acid) inhibition occurred during the reaction, regardless of the media used. In order to characterize the kinetics, constants of the reaction (K_M, v_{max}, and K_I) were determined.

Esters of short- and medium-chain acids and alcohols are important flavour components, which are considered as natural if the initial compounds are natural and enzyme is used as a catalyst. These flavour components can be synthesized by microbial lipases (E.C. 3.1.1.3. triacylglycerol acylhydrolase) as it was presented by *Leblanc et al.* [1], however the yields of ethyl and methyl acetate were found as the lowest [2] regardless of the lipase preparation used.

Natural ethyl acetate has been manufactured enzymatically by using a Lipozyme IM® lipase preparation in organic solvent (hexane), so far [3—5]. Nowadays, however, the trend goes towards "more natural" compounds, *i.e.* the natural products should be free of solvent traces, as well. In our project the esterification by another lipase preparation was carried out in both organic solvent and solvent-free system, and the kinetical results were compared.

Kinetics of enzymatic esterifications has been studied by several scientists. Many of them found that esterification reaction of long-chain acids and short-chain alcohols was inhibited by the alcohol compound of the reaction, regardless whether the reaction was carried out in organic solvent (even in supercritical solvent) or in solvent-free system. Some examples from the relevant papers are listed in Table 1. On the other hand, acid inhibition was observed during esterification of short-chain acids and longer-chain alcohols, as it was presented in some papers, summarized also in Table 1.

In this work the particular purpose was to decide which substrate shows stronger inhibition effect in the esterification reaction of ethanol and acetic acid (both short-chain components) in both solvent-applying and solvent-free systems. Therefore a series of experiments on esterification by lipase enzyme was designed and carried out to obtain detailed kinetical data (progress curves, initial reaction rates).

EXPERIMENTAL

Novozyme 435® lipase preparation from Novo Nordisk (Denmark) was used, manufactured by recombinant DNA technology. The gene coding for the lipase has been transferred from a selected strain of *Candida antarctica* to the host organism, *Aspergillus oryzae*. The enzyme produced is immobilized onto a macroporous acrylic resin, diameter 0.3—0.9 mm, its reported activity is 7000 PLA g^{-1} (PLA means propyl laurate activity and its unit is defined as 1 μmol propyl laurate formed per minute per gram of catalyst under standard conditions [12]). The initial compounds for the reaction were natural acetic acid and ethanol (both anal. grade) purchased from Daniel (Germany) and Győri Szeszgyár és Finomító RT (Hungary), respectively. The organic solvent used was heptane (anal. grade) from Reanal (Hungary).

Esterifications were carried out in shaking flasks containing 25 cm^3 of reaction mixture and 0.2 g of enzyme preparation. The initial water content of all the reaction mixtures was adjusted to 0.50 mass % and checked carefully. The flasks were shaken at 150 min^{-1} and 40 °C temperature in a New Brunswick G24 incubator shaker.

10 mm^3 of samples were taken in duplicate and analyzed without any further treatment. Ester concentrations in the samples were determined by gas chromatography, using Hewlett—Packard Model 5890A instrument equipped with a flame ionization detector, a 25 m FFAP fused silica capillary column (Macherey Nagel, Germany), and a capillary inlet system fitted with a split line that allows the nitrogen flow to be

Table 1. Types of Substrate Inhibitions in Esterification Reactions by Lipase

Substrate, acid	Substrate, alcohol	Solvent	Enzyme	Inhibition observed	Ref.
Palmitic acid	Isopropyl alcohol	No data	Lipozyme IM	Alcohol	[6]
Palmitic acid	Isopropyl alcohol	Solvent-free	Novozyme 435	Alcohol	[7]
Fatty acids	Sulcatol (6-methyl-5-hepten-2-ol)	Hexane	Immobilized *Candida rugosa*	Alcohol	[8]
Oleic acid	Ethanol	Supercrit. CO_2	Lipozyme IM	Alcohol	[9]
Acetic acid	Geraniol	No data	Surfactant-coated lipase	Acid	[10]
Acetic acid	Isopentyl alcohol	Heptane	Lipozyme IM	Acid	[11]

split at 60:1. Nitrogen flow rate through the column was 2.0 cm^3 min^{-1}. Injection port and detector temperatures were 225 °C. The column temperature was held at 50 °C for 2 min and then heated to 150 °C with a rate of 10 °C min^{-1} and held there for 5 min.

The water content of the reaction mixtures was determined by Mettler DL 35 automatic Karl-Fischer titrator.

RESULTS AND DISCUSSION

Experiments in heptane solvent were carried out with various initial acid (substrate) concentrations (S) in the range of 0.05 mol dm^{-3} and 4.20 mol dm^{-3} always for four different initial ethanol concentrations. The amounts of ester produced as a function of reaction time (progress curves) were determined. The initial reaction rates (v_o) for the ester production were calculated from the first part of the progress curves, where conversions were always below 10 % (in higher initial concentrations it was below 2 %). The calculations were performed in a way that water concentration – initially 0.50 mass % – never reached 0.55 mass %, otherwise it would have influenced the reaction rate and should have been removed continuously (as we published it earlier [5]). Thus effects of products present (especially water) could be eliminated. The reaction rates as a function of initial substrate (acid) concentrations are presented in Fig. 1.

As it can be seen, all of the curves of reaction rate *vs.* acid concentration have a maximum, but their heights and positions are different. Thus the effect of different initial ethanol concentrations used is considered two-fold. The maximal initial reaction rates determined from the progress curves are shifted towards higher initial acid concentrations as the ethanol content increases, on one hand. Among the summits, the highest maximum is observed at 4.4 mol dm^{-3} ethanol concentration, on the other hand. It means that the increasing ethanol content seems to "defend" the enzyme from the "harmful" acid, thus the lipase preparation is able to work more effectively in higher and higher acid concentrations. However, acid inhibition effect can be observed in every reaction rate *vs.* substrate concentration curve, although it occurred at higher and higher acids concentrations as the amount of ethanol present increased.

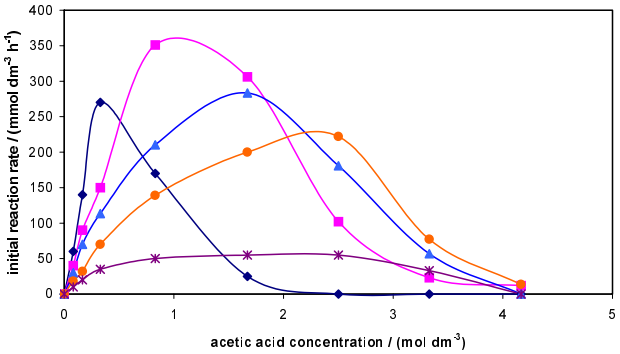

Fig. 1. Initial reaction rates *vs.* substrate (acid) concentrations in heptane organic solvent and in solvent-free media. Initial ethanol concentrations: ♦ 2.2 mol dm^{-3}, ■ 4.4 mol dm^{-3}, ▲ 7.6 mol dm^{-3}, ● 10.9 mol dm^{-3}, × solvent-free.

Based on the data obtained, now it is clear that acid inhibition occurs in the particular reaction. According to our experimental results for esterification of short-chain acids and alcohols, the effect of acid is stronger and more harmful towards the enzyme than that of the alcohol.

As a consequence of these results, it seemed reasonable to perform more experiments where ethanol concentration is increased further on, reaching finally a point where no organic solvent is present in the mixture. In other words, excess of ethanol "substitutes" for the organic solvent.

Experiments in solvent-free system were carried out with various initial acid concentrations (in the range of 0.05 mol dm^{-3} and 3.5 mol dm^{-3}) in solvent-free media. In the two-component system (acetic acid and ethanol are present) the initial alcohol concentration varies when the value of acid concentration varied. Thus there is a fundamental problem to set a given initial alcohol concentration as it was presented in case of the solvent-using systems.

Table 2. Kinetic Constants

Initial ethanol conc.	v_{max}	K_M	K_I
mol dm^{-3}	mol dm^{-3} h^{-1}	mol dm^{-3}	mol dm^{-3}
2.2	0.55	0.60	0.29
4.4	0.57	0.87	1.01
7.6	0.49	1.07	1.26
10.9	0.37	1.41	1.90
Solvent-free system	0.12	0.68	1.73

From the progress curves (concentrations of ester formed as a function of reaction time) the initial reaction rates were determined. The values obtained were plotted against initial acid (substrate) concentration (Fig. 1).

It can be seen that beyond 0.8 mol dm^{-3} substrate concentration the reaction rate has reached a maximal value, a plateau. However, the rate has not remained constant, but started to decrease approximately at 2.8 mol dm^{-3} acid concentration. It means that substrate (acid) inhibition has occurred here, as well.

Comparing the data obtained in solvent-free system and heptane, the reaction rates determined in the organic solvent were found much higher than those measured in the solvent-free system. Moreover, the acid concentration values, where maximal reaction rates were observed in heptane, cover the range where the initial reaction rate values in the solvent-free system have formed the plateau.

Calculating the Michaelis—Menten parameters, in both systems substrate (acid) inhibition was found to occur during the esterification of acetic acid and ethanol by lipase. Since the enzyme used was an immobilized preparation, all of these kinetic phenomena should be carefully handled. To estimate the (in)significance of the diffusion limitation, the time constants for the reaction (t_r) and the diffusion (t_d) were compared. Under the circumstances of the experiments, $t_r \gg t_d$ was found implying that the reaction was not influenced significantly by mass transfer, thus the overall rates observed are considered as kinetically controlled.

Characterizing the enzymatic reaction, the process should be considered as a two-substrate (acid and alcohol), two-product (ester and water) reaction. To simplify the kinetical approach, however, only acid was taken as substrate, since its role seemed more significant, moreover from the engineering and procedure points of view this simplification makes the reaction design easier.

For the description of the reaction kinetics, the modified Michaelis—Menten equation was used, as follows

$$v_o = \frac{v_{max} S}{K_M + S + S^2/K_I}$$

where K_M is the Michaelis—Menten constant, v_{max} is the maximal reaction rate, while K_I is the inhibition constant.

This equation contains three parameters, which can be determined for the particular initial reaction rate *vs.* substrate concentration curve either by graphical methods (*e.g.* Lineweaver—Burk linearization) or by numerical methods [13, 14]. Using the increasing sections of the rate *vs.* acid concentration curves (up to the maximums), the values of K_M and v_{max} were determined by graphical method (double reciprocal transformation). Then values of K_I constants were determined by a numerical method (parameter estimation by the Nelder—Mead type simplex search) applying these data as initials. The results of the determinations for both the solvent-applying and the solvent-free systems are summarized in Table 2. The reliability of the values calculated was always within 7 %.

It can be seen that the trend of v_{max} values is following the trend of the peaks of the reaction rate *vs.* acid concentrations curves and the highest v_{max} was found at 4.4 mol dm^{-3} initial ethanol concentration. K_M values are increasing gradually with the growing ethanol concentrations up to 10.9 mol dm^{-3}, which means that – if no acid inhibition occurred – the (half of the) maximal reaction rates would be reached at higher and higher substrate concentrations.

The values of K_I are increasing with the growing ethanol concentrations, too, which implies that the sections with no apparent substrate (acid) inhibition are extended applying more and more ethanol in the initial mixture. These data have confirmed our concept on the peculiar "enzyme defending" role of ethanol described above.

Regarding the solvent-free system, it can be observed that the obtained data of K_M and K_I are located within the range determined for the solvent system, on one hand. The v_{max} is, however, much lower than those obtained in the solvent-applying system, on the other hand, probably due to the slower reactions measured at the solvent-free system.

CONCLUSION

The kinetics of enzymatic esterification of ethanol and acetic acid in heptane solvent and solvent-free media was investigated and it was found that acid (substrate) inhibition occurred and presence of ethanol

seemed to "defend" the enzyme. It means that acid inhibition has taken place at higher initial acid concentrations at increasing ethanol content. As a consequence, lipase catalyzed, solvent-free esterifications of short-chain alcohols and acids are worth realizing in alcohol excess (to substitute the organic solvent). The whole reaction was characterized by the determined Michaelis—Menten constants.

The data obtained in this work help to choose the most suitable reactor system (including the coupled continuous water removal) and to determine the proper reaction parameters, hence this study was useful to elaborate a technology for pilot or semi-pilot production of natural ethyl acetate.

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