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Kinetics of Gas-Phase Hydrolysis of Ethyl Acetate Catalyzed by Immobilized Lipase

Victor H. Perez,*[,1] Everson A. Miranda,[2] and Gustavo P. Valença[3]

Departments of [1],[2]Biotechnological Processes and [3]Chemical Processes, School of Chemical Engineering, State University of Campinas, PO Box 6066, 13083-970, Campinas-SP, Brazil, E-mail: victorh@fea.unicamp.br

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Abstract

Reactions catalyzed by supported enzymes present important advantages when compared with those in aqueous media or organic solvents: separation of enzymes from substrate is easily accomplished, enzyme stability may be improved, and control of the reaction products is more accurate. We present the experimental results of the kinetic study of ethyl acetate hydrolysis in gaseous phase catalyzed by a commercial immobilized lipase (Lipozyme IM; Novo Nordisk). The hydrolysis reaction was studied as a function of ethyl ester and water partial pressure at a constant temperature of 318 K. The amount of biocatalyst used was varied between 100 and 300 mg, and the reaction was studied in a flow-through glass microreactor. Under the conditions used, water was an important parameter in the gas-phase reaction. Activation energy was 24.8 kJ/mol and the overall order of reaction was one. Finally, a Bi-Bi reaction mechanism is proposed.

Index Entries: Biocatalysis; enzymatic hydrolysis; supported enzymes; lipases; gas-solid.

Introduction

Enzymes have been used as catalysts mostly in aqueous phase. Organic solvents have been used to solubilize substrates and products, but for such systems the enzymes are not stable and may be inhibited by reaction products *(1)*. However, enzymes in organic solvent systems are much less prone

*Author to whom all correspondence and reprint requests should be addressed.

to microbial contamination *(2,3)*. Despite this advantage, loss of enzymatic activity may be a severe drawback. In 1980, however, it was observed that when a small amount of water is added to the system enzymes in organic solvents may have activity similar to that in aqueous phase *(4–6)*. Enzymes in organic solvents also have limitations, although, such as denaturation, formation of undesirable products, and the need for high enzyme concentrations *(1,7)*.

A recent advance in enzyme catalysis is the use of solid-phase enzymes as catalysts for gas-phase reactions. Enzymes in solid phase may be self-supporting microbial cells, a bed of enzyme particles (referred to in this article as unsupported enzymes), or enzymes immobilized on solids (organic or inorganic). According to Bárzana *(8)*, the pioneering studies on gas-solid enzymatic reactions date to 1969 *(9)*, when dried cells were used. Other researchers also used this system to study catalytic gas-phase reactions *(10–13)*. The advantages of this type of system have been thoroughly reviewed and emphasized *(14)*. For example, there is no need for covalent immobilization of the enzymes. In addition, as the biocatalyst is in the solid phase it may be recycled, and, thus, batch or continuous reactors may be used. Because the reaction takes place in the gas phase, where diffusion coefficients are large, it is less prone to diffusion limitations. Separation of the products and substrates from the catalyst is simplified, a step that is usually very expensive in biotechnological applications. Substrate separation may be achieved by simple unit operations such as condensation. Generally, enzymes in aqua-restricted systems are more stable than enzymes in solution. Finally, the water activity may be easily controlled in gas-phase reactions, thus allowing no conventional reactions such as synthesis or transfer reactions using hydrolases to occur. Some potential applications of gas-phase enzymatic reactions include removal of toxic gases such as nerve gas *(15)* from gaseous streams; development of biosensors; and synthesis of pharmaceutical chemicals, fragrances, and aromas. However, the boiling points of most substrates are higher than 523 K at atmospheric pressure *(16)*, thus limiting the use of gas-phase enzymatic reaction.

Most studies with gas-phase enzymatic reactions have emphasized the water-enzyme interaction and the influence of water on enzyme activity and stability *(1,14,15,17–26)*. Recently, gas-phase enzymatic reactions were studied in order to identify the most relevant steps in the reaction and to determine individual rate constants and thermodynamic parameters *(26–29)*.

Although the gas-phase enzymatic reaction is a promising area for technological advancement, it is necessary to obtain further information about these systems. For example, it is not clear whether gas-phase enzymatic reactions take place through the same sequence of steps as liquid-phase reactions. Thus, the objective of the present work was to study the kinetics of gas-phase reaction using ethyl acetate hydrolysis catalyzed by a supported lipase as a reaction model to obtain a rate expression according to enzyme/substrate interaction mechanics. All the calculations and

discussion presented in this article were based on conversion values corresponding to 1.5 h of reaction, when the stationary state was reached.

Materials and Methods

Biocatalyst

The biocatalyst was the commercial lipase Lipozyme IM from Novozymes (Araucária, Paraná-Brazil). The lipase in Lipozyme IM was produced by *Mucor miehei*, whose genetic code was transferred to *Aspergillus oryzae*. The enzyme was immobilized on a phenolic macroporous ion-exchange hydrophilic resin (Duolite 568) *(30)*. The biocatalyst particle size was between 0.2 and 0.6 mm and the apparent density between 350 and 450 kg/m^3, according to the manufacturer.

Reaction Setup

The reaction rig for the gas-phase reactions was made of Pyrex® glass and copper tubing and had two independent gas lines: one for ethyl acetate ($CH_3COOCH_2CH_3$) and the other for water (H_2O) (Fig. 1). Approximately 50 mL of a liquid reactant ($CH_3COOCH_2CH_3$ or H_2O) was added to each reservoir. The carrier gas, He, flowed continuously (40 mL/min), first through the liquid reservoir (*see* in Fig. 1A) and then through the saturator (*see* Fig. 1B). The gas mixture leaving each saturator was He saturated with $CH_3COOCH_2CH_3$ or H_2O with the reactant vapor pressure corresponding to the temperature in each saturator. The saturators were connected to the mixing chamber (*see* Fig. 1C), from where the gas mixture was sent to the fixed-bed reactor (1.0 cm internal diameter [ID] and 5.0 cm length) (*see* Fig. 1D) containing about 0.150 g of catalyst. The system was connected to a gas chromatograph (*see* Fig. 1E) for reactant and product analysis. All flow rates were measured at room temperature and ambient pressure (93.5 ± 0.026 kPa). The gas mixture in a 5.0-mLloop containing ethanol, acetic acid, ethyl acetate, water, and carrier gas was sampled with a six-way valve maintained at 475 K. Samples were analyzed by a gas chromatograph (CG-35) equipped with a Poropak Q packed column (80/100 mesh, 3 m × 0.3 cm ID; Chromacrom, São Paulo, Brazil) and a thermal conduction detector (TCD) detector. Data were collected, stored, and analyzed using Borwin Data Acquisition Software 2.1. The injector was maintained at 393 K, the column at 453 K, and the detector at 473 K. Calibration curves for reactants and products were prepared by changing the saturator temperature in order to obtain different vapor pressures for each substance. The vapor pressure, P_{vap}, was calculated for each substance using Antoine's equation:

$$\log P_{vap} = A - \frac{B}{T + C} \quad (1)$$

in which T is the saturator temperature (K); P is the vapor pressure (Pa); and A, B, and C are constants. The retention times for H_2O, CH_3CH_2OH, CH_3COOH, and $CH_3COOCH_2CH_3$ for the experimental conditions used were 2.3, 4.4, 13.4, and 15.0 min, respectively.

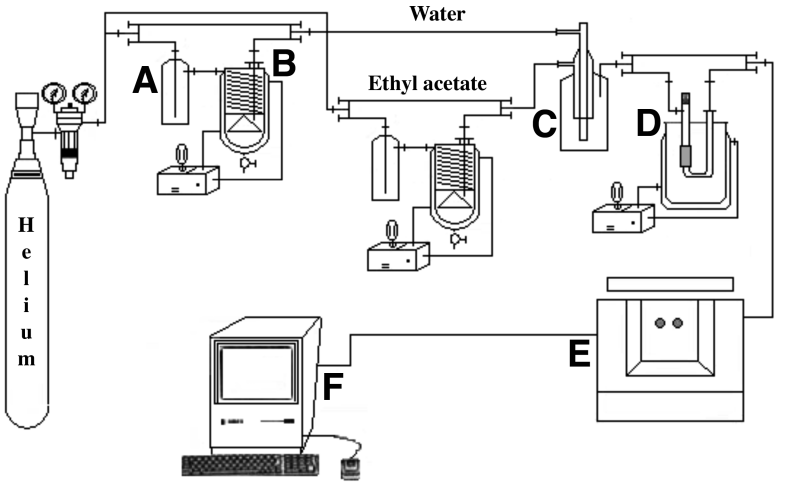

Fig. 1. Reaction setup: **(A)** reactant reservoir; **(B)** temperature-controlled saturator; **(C)** mixing chamber; **(D)** reactor; **(E)** analyzer; **(F)** measurement and temperature control and data acquisition.

Reaction Conditions

Ethyl acetate conversion was maintained less than 10% to avoid heat and mass transfer limitations during reaction. The average rate of reaction, $r_{A,}$ in moles of converted substrate per hour per milligram of biocatalyst was calculated using the following equation for differential reactors:

$$-r_A = \frac{F_{A0}X}{Wt} \tag{2}$$

in which X is the conversion of ethyl acetate, Wt is the mass of biocatalyst, and

$$F_{A0} = \vartheta C_{A0} \tag{3}$$

is the molar flow rate and is equal to the volumetric flow rate, ϑ, times the substrate concentration, C_{A0}, measured at the reactor exit. Prior to reaction, water-saturated He flowed through the bed of biocatalyst for 30 min at room temperature. The reactor was then isolated from the gas current, and the reactor temperature was increased to the reaction temperature. The flow of He was then switched to the liquid reservoir containing ethyl acetate. He flowed for at least 30 min in order for the system to achieve steady state. Finally, the gas current containing both ethyl acetate and water was switched to the reactor and the reaction started. Gas samples were taken at 20-min intervals, and the reaction was run for at least 2 h.

Results and Discussion

Reproducibility

To test the reproducibility of the experimental system for this reaction, the reaction was carried out three times at a specific set of conditions with

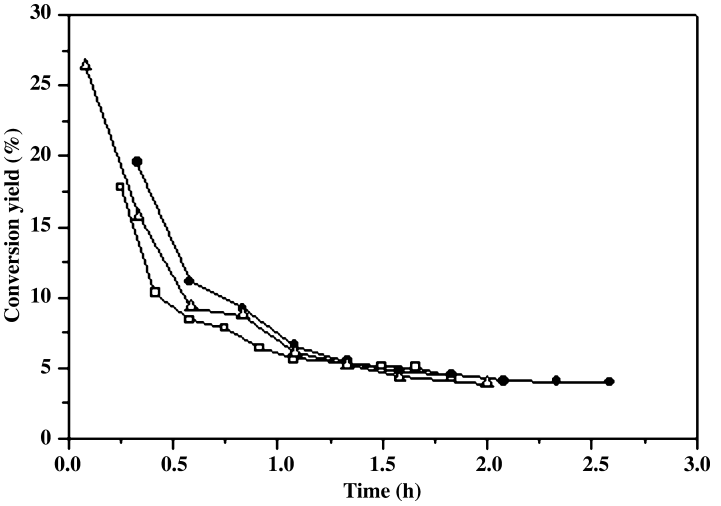

Fig. 2. Ethyl acetate hydrolysis with 150.0 mg of catalyst, total volumetric flow rate of 50 mL/min, and reaction temperature of 318 K. Ethyl acetate partial pressure was 2032 Pa and water partial pressure was 609 Pa.

the same biocatalyst sample (Fig. 2). In all experiments a drop in ester conversion was observed during the first 60 min, irrespective of the experimental conditions. Comparison on the basis of the initial conversion was not possible because the reactor needed between 1 and 1.5 h to be stabilized (transient phase). This stabilization phase was owing to the equilibrium of the biocatalyst hydration state, and all reaction rates (conversions yield) were measured under steady-state conditions. On the other hand, transient state has been reported depending on the bioprocess setup *(13,25)*.

All experiments in the present study were reproducible, within the experimental error for kinetic measurements (within 20%). The standard deviation (SD) of the kinetic data taken at different reaction times was always less than 10% of the average conversion. After steady state was reached (1.3 h), however, the average conversion was 4.4% with an SD of 9×10^{-3}.

To study the decrease in conversion with time, the biocatalyst was exposed to a water-saturated He flow for 30 min at room temperature prior to the ester hydrolysis reaction. The reactor was closed and its temperature raised to reaction temperature. Water-saturated He was then flowed through the reactor and the gas stream sent to the gas chromatograph. The amount of water in the gas current decreased with time (Fig. 3) in the same way as in the ethyl ester conversion, suggesting that the decrease in ester conversion is owing to a lower water activity in the system.

External Mass Transfer Limitations

To test the reaction conditions for mass transfer limitations, the volumetric flow rate, substrate concentration (partial pressure), and reaction

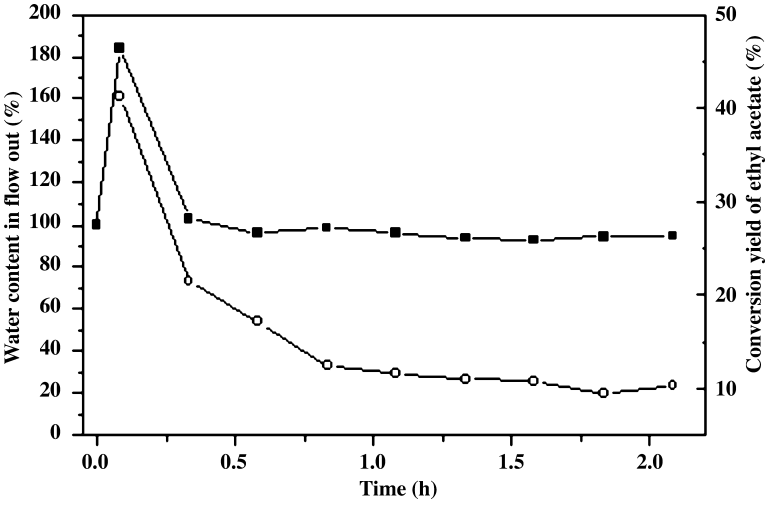

Fig. 3. Water flow rate out of biocatalyst bed (■) compared to conversion of ethyl acetate (○). Reactant partial pressure in the feed was 2338 Pa for water and 1898 Pa for ethyl acetate. Total gas flow rate and reaction temperature were 54 mL/min and 318 K, respectively.

temperature were maintained constant and equal to 52.5 mL/min, 1898 Pa for ethyl acetate and 609 Pa for water, and 318 K. The catalyst mass was then reduced by half (from 153.3 to 77.5 mg), and the conversion of ethyl acetate was measured (Fig. 4). A decrease in the biocatalyst mass resulted in a decrease in conversion from 26.4 to 13.3% at the beginning of the reaction, suggesting that the reaction occurred in the absence of mass transfer limitations. However, after 30 min the conversion of ethyl acetate on 77.3 mg of biocatalyst was approximately constant (between 4 and 7%) and equal to the conversion on 153.3 mg of biocatalyst.

At first glance it may seem that after 30 min the reaction system may become mass transfer limited. However, since the volumetric flow rate is maintained constant and the catalyst mass is reduced by half, the modified space velocity, F/W, is doubled. Since the amount of water is also kept constant, more water is available to the enzyme in the second case. Since enzyme activity and stability depend on the amount of water in the system, doubling the water may double the specific activity of the enzyme.

Biocatalyst Hydration Time

Three samples of biocatalyst were hydrated for different lengths of time, 0.5, 1, and 2 h, at a water partial pressure of 2338 Pa at 318 K. The hydrolysis reaction of ethyl ester was then carried out on each biocatalyst under the same reaction conditions (Fig. 5). The hydration time between 0.5 and 2 h had no significant influence on the conversion, suggesting the saturation of the biocatalyst with water.

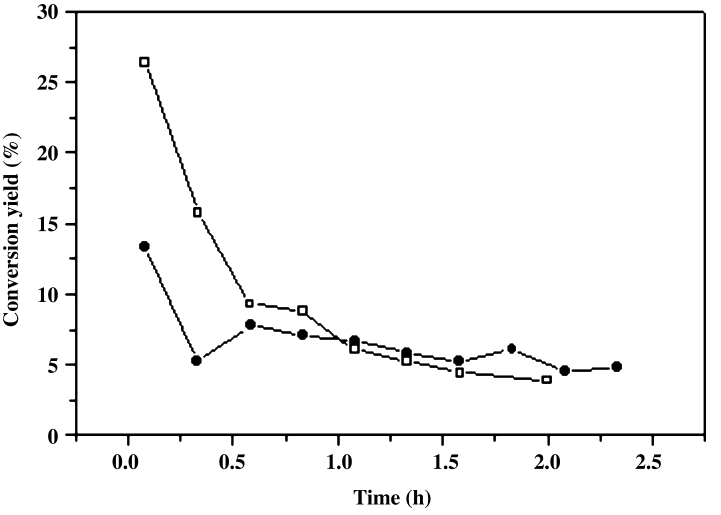

Fig. 4. Influence of biocatalyst mass on conversion of ethyl acetate: (□) 153.3 mg; (●) 77.5 mg. Total volumetric flow rate, substrate concentrations (partial pressure), and reaction temperature were 52.5 mL/min, 1898 Pa for ethyl acetate and 609 Pa for water, and 318 K, respectively.

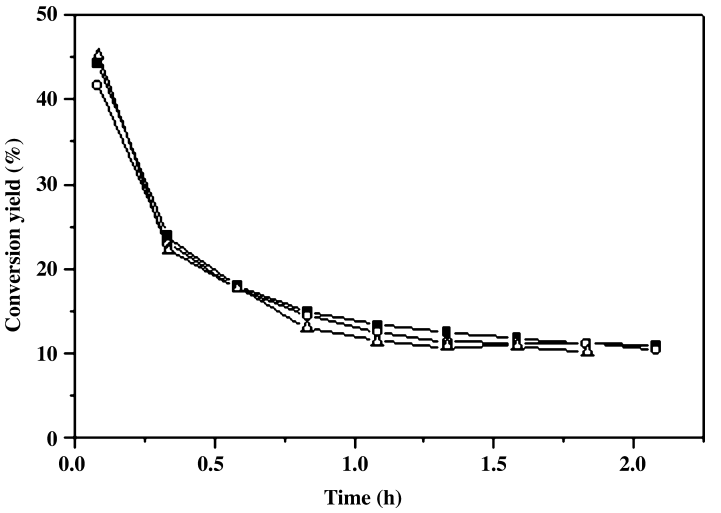

Fig. 5. Influence of biocatalyst hydration time on ethyl acetate hydrolysis conversion. All three biocatalyst samples were hydrated in a continuous flow of He saturated with water with a partial pressure of 2338 Pa: (■) 0.5 h; (●) 1 h; (▲) 2 h. Total volumetric flow rate, reaction temperature, and ethyl acetate partial pressure were 54 mL/min, 318 K, and 1898 Pa, respectively.

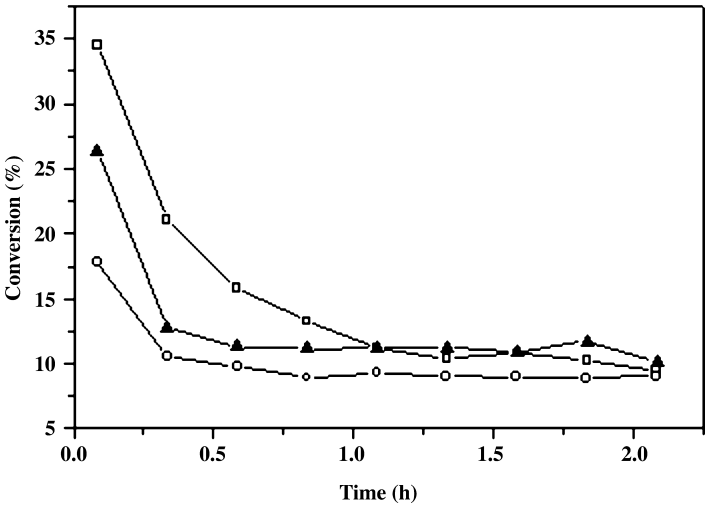

Fig. 6. Stability of biocatalyst during hydrolysis of ethyl acetate on same sample of biocatalyst. All hydration steps were in a continuous flow of He saturated with water with a partial pressure of 2338 Pa: (□) first reaction after hydration for 30 min; (○) second reaction after hydration for 30 min; (▲) third reaction after hydration for 15 h. Total volumetric flow rate, reaction temperature, and partial pressure of ethyl acetate were 54 mL/min, 318 K, and 1898 Pa, respectively.

Stability of Biocatalyst

The stability of the biocatalyst was studied with three reaction runs with the same sample of biocatalyst under fixed reaction conditions. In the first run, 150 mg of biocatalyst was hydrated for 30 min in a flow of He saturated with 2338 Pa of water prior to reaction. The reaction was then run for 2 h. After the first run, the biocatalyst was rehydrated for 30 min under the same conditions as those of the first run and another reaction run was started and maintained for 2 h. Finally, the biocatalyst was hydrated for 15 h and used in a third reaction run for 2 h (Fig. 6). The conversion of ethyl acetate in the second run was always smaller than that in the first run. However, in the third run, after a long hydration time, the steady-state conversion was similar to that in the first run. Thus, the decrease in conversion in the second run was the result of something other than deactivation.

At this point, we can only speculate on the reason for the decrease in conversion. During reaction, the peaks for ethyl acetate, ethanol, and water were always observed in the chromatogram. The number of moles of ethanol calculated from the peak area was equal to that of reacted ethyl acetate, within the experimental error. The peak for acetic acid, however, was clearly observed only for the first point. The number of moles of acetic acid calculated from the peak area for subsequent points was always smaller than that calculated from the amount of converted ethyl acetate, suggesting that acetic acid was accumulating on the support or somewhere else in the system. Since at the levels of conversion used in this work the vapor pres-

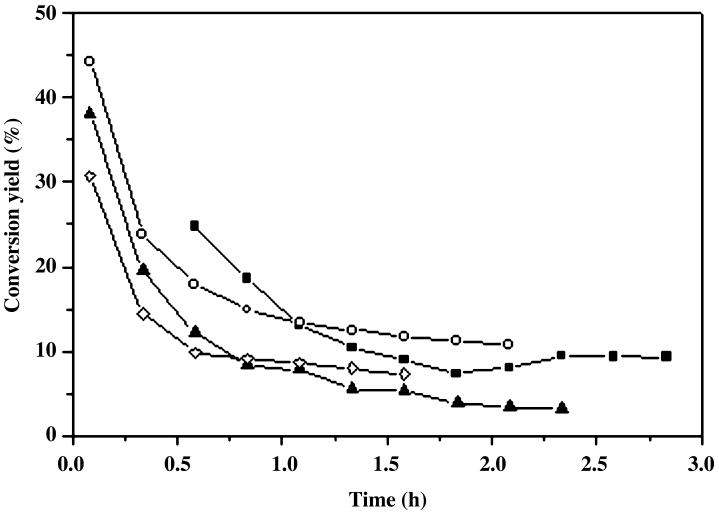

Fig. 7. Influence of temperature on rate of hydrolysis of ethyl acetate: (△) 308 K; (○) 318 K; (■) 328 K; (◆) 338 K. Total gas flow rate was 54.0 mL/min and 150 mg of biocatalyst. The partial pressure of ethyl acetate was 1898 Pa and that of water was 2338 Pa.

sure of the acetic acid was always lower than its saturation pressure, no condensation was expected. In addition, the system line downstream from the reactor was maintained at 400 K to prevent water condensation. Thus, the acetic acid formed was probably adsorbed on the biocatalyst, most probably on the support, an anionic ion-exchange resin. The long rehydration treatment of the biocatalyst therefore may have removed some of the acid that accumulated during the reaction, making conversion of the ethyl acetate in the hydrolysis reaction larger than in the second run.

The stability of the biocatalyst was also studied at different temperatures: 308, 318, 328, and 338 K (Fig. 7). In all experiments the total gas flow rate was 54.0 mL/min, and the partial pressure of ethyl acetate was 1898 Pa and that of water was 2338 Pa. The data in Fig. 7 suggest that no denaturation of the enzyme occurred for all temperatures used, since the conversions after 1.5 h were constant for a experiment at a specific temperature. As the reaction temperature was increased from 308 to 318 K the reaction rate increased. However, after 1.5 h the reaction rate decreased as reaction temperature increased above 318 K. This decrease in reaction rate was probably owing to a decrease in the hydration of the biocatalyst resulting from a decrease in water activity. Yang and Russell *(1)* and Kim and Rhee *(31)* observed an increase in conversion as reaction temperature increased from 298 to 313 K for gas-phase enzymatic reactions. However, they observed that above 323 K the enzyme denatured. Parvaresh et al. *(25)* obtained similar results for the reaction of transesterification with lipase and cutinase. They suggested that the combined effect of temperature and decrease in water activity should be taken into account to explain the results. Boy et al.

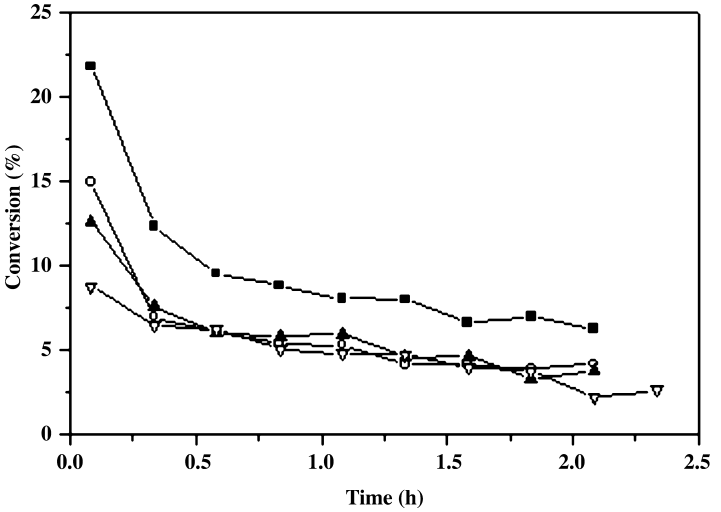

Fig. 8. Ethyl acetate conversion during hydrolysis as function of time for water partial pressure of 609 Pa and different partial pressures of ester: (■) 898 Pa; (○) 3333 Pa; (▲) 5653 Pa; (▽) 9746 Pa. Total volumetric flow rate was 54 mL/h and reaction temperature was 318 K.

(32) presented similar results for other enzymes, including the biocatalyst used in the present work, for aqueous-phase reactions. In their study, the enzyme activity decreased above 313 K.

Influence of Substrate Partial Pressure on Reaction Rate

The influence of substrate partial pressure on the reaction rate was studied by maintaining one substrate partial pressure constant and varying the other (Figs. 8 and 9, Table 1). The values of water partial pressure were 610, 1228, and 2338 Pa and of ethyl acetate were 1898, 3333, 5653, and 9746 Pa. For a given value of water partial pressure, the conversion first decreased as the ester partial pressure increased from 1898 to 3333 Pa, remaining constant thereafter as the ester partial pressure increased to 9746 Pa (Fig. 8), suggesting a possible substrate or product inhibition of the reaction.

On the other hand, in the gas phase water has a negative effect on the stability but a positive effect on the enzyme activity *(18)*. Furthermore, in the hydrolysis, the reaction rate is favored at high water activity because it was observed that the conversion increased slightly as the water partial pressure increased for a given ester partial pressure (Table 1). As the ester-to-enzyme ratio increased, the ester conversion decreased but the amount of gas-phase ethanol increased (Fig. 9).

The inhibition could be explained by different reasons. For example, the polarity of the microenvironment of the lipase was modified. The hydrophilicity of the organic phase increases while increasing ethyl alcohol and acetic acid concentration. Both substrates have certain polarity and, there-

Table 1
Rate of Reaction for 1.5 h

Water partial pressure (Pa)	Ester partial pressure (Pa)	Fitting equation for conversion: $0.0833 \le t_{reaction} \ge 2.0833$ (h)	Ester conversion (%)	Rate of reaction $\times 10^4$ (mol/[h·mg])
610	1898	$7.2 + 19.7e^{(-t/0.27)}$	7.20	1.20
	3333	$4.5 + 15.5e^{(-t/0.21)}$	4.50	1.31
	5653	$4.2 + 10.1e^{(-t/0.37)}$	4.40	2.18
	9746	$0.99 + 7.7e^{(-t/1.54)}$	3.90	3.31
1228	1898	$8.7 + 28.6e^{(-t/0.24)}$	8.70	1.45
	3333	$5.8 + 20.9e^{(-t/0.24)}$	5.70	1.70
	5653	$3.7 + 12.7e^{(-t/0.39)}$	3.90	1.95
	9746	$3.8 + 6.7e^{(-t/0.48)}$	4.10	3.50
2338	1898	$10.4 + 41.2e^{(-t/0.28)}$	10.60	1.76
	3333	$6.3 + 29.3e^{(-t/0.26)}$	6.40	1.87
	5653	$4.3 + 23.3e^{(-t/0.31)}$	4.50	2.22
	9746	$5.4 + 11.9e^{(-t/0.15)}$	5.40	4.62

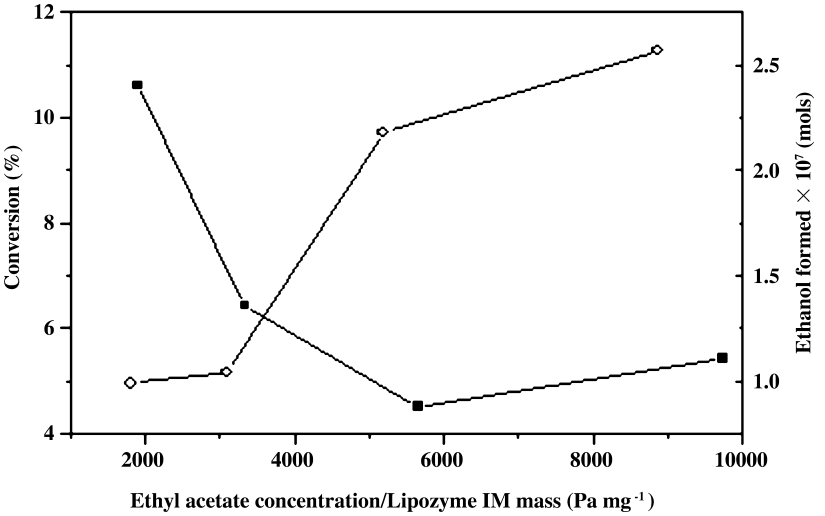

Fig. 9. Ester conversion and formation of ethanol as function of ethyl acetate concentration on mass of biocatalyst ratio in hydrolysis of ethyl acetate reaction at 318 K after reaction for 1.5 h: (■) conversion; (○) amount of ethanol. Total volumetric flow rate was 54 mL/min and water partial pressure was 2338 Pa.

fore, the ability to bind water molecules and damage the water layer around lipase, which is more severe in systems without organic solvent because concentrations of both substrates in the enzyme microenvironment are higher. Consequently, "drying" of the biocatalyst causing inactivation is possible. In addition, acetic acid may act as a competitive inhibitor and cause lipase deactivation by a drop in pH.

The reaction rate was calculated by fitting the experimental ester conversion data (X) vs time with an exponential function, using the Microcal Origin 5.0 Professional program:

$$X = D + H \exp\left(\frac{-t}{J}\right) \tag{4}$$

in which t is the reaction time (h), and D, H, and J are constants assuming a differential mode of operation for the reactor. Table 1 provides the values of water and ethyl ester partial pressures, the fitting equation parameters, and the values for the estimated conversion and reaction rate at 1.5 h.

In none of the equations (Table 1) is the conversion larger than 100% ($D + H$, for $t = 0$), usually varying between 9 and 52%. A simplified kinetic model was then adjusted to the calculated values of conversion for $t = 1.5$ h as follows:

$$-r_A = kPi_W^{\alpha} Pi_e^{\beta} \tag{5}$$

in which k is the rate constant (mol/[h·mg·Pa]); Pi_w and Pi_e are the partial pressures of water and ester (Pa), respectively; and α and β are the orders of reaction regarding water and ester, respectively.

The data in Table 1 were fitted to Eq. 5 using Polymath version 4.0. The sum of the squares of the residuals (SQR) was used as the best choice for the model. The results were $k = 4.5 \times 10^{-11}$ mol/(h·mg·Pa), $\alpha = 0.25$, $\beta = 0.74$, and SQR = 1.3×10^{-8}.

The order of reaction with respect to ester (β) is 0.7 and with respect to water (α) is approx 0.3. The low value for the order of reaction with respect to water may reflect two competing phenomena of water in this reaction: as a reactant and as a necessary agent for the enzyme activity (enzyme hydration).

After determining the kinetic data for the reaction, a sequence of elementary steps was proposed and the values of the constant were obtained. Since in the hydrolysis of ethyl acetate there are two substrates, a Bi-Bi mechanism was proposed (Fig. 10). In the proposed mechanism, ethyl acetate, A, and water, W, may be bound randomly to the enzyme. However, once one substrate binds to the enzyme the binding of the second substrate is affected.

This sequence of steps cannot be described by a Michaelis-Menten equation *(33)*. According to Segel *(34,35)* Bi-Bi reactions may be studied assuming a fast equilibrium between substrates and the enzyme, followed by decomposition of the enzyme-substrate complex (EAW) (Fig. 10). For example, a ping-pong Bi-Bi sequence of steps was used to explain the results of alcoholysis catalyzed by *Candida antarctica* lipase B *(27)*.

A fast equilibrium between substrate and enzyme was assumed for the reaction. It was also assumed that the rate-determining step is the decomposition of complex EAW, represented by

$$EAW \xrightarrow{Kp} E + P$$

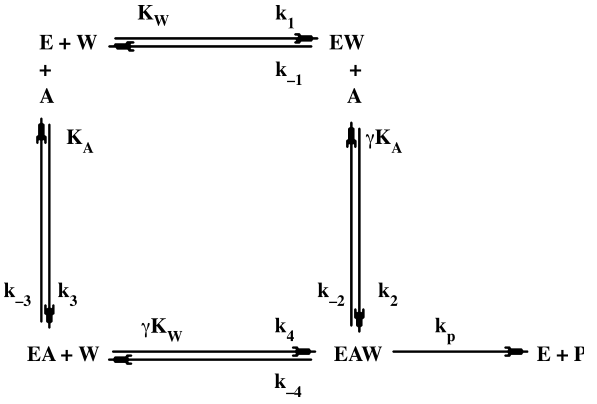

Fig. 10. Reaction scheme for Bi-Bi type of sequence of steps.

The rate expression for the scheme depicted in Fig. 10 is given by

$$r_A = \frac{k_0 \exp^{-E_a/RT} [A] [W]}{(\gamma K_A K_W + \gamma K_A [W] + \gamma K_W [A] + [A] [W])} \quad (6)$$

in which k_0 is the preexponential factor; E_a is the activation energy (kJ/mol); T is the reaction temperature (K); R is the gas constant; (A) and (W) are the partial pressures of water and ester (Pa), respectively K_A and K_W are the equilibrium constants (Pa); and γ is the factor that expresses the relation between the bound substrate and the other substrate. Equation 6 was fitted by a linear equation using Polymath version 4.0. The values for the reaction parameters were $k_0 = 23.19$ mol/(h·mg), $E_a = 24.8$ kJ/mol, $K_A = 2493$ Pa, $K_W = 91$ Pa, and $\gamma = 12.3$. The SQR was 1.742×10^{-8} with an approximate error of 10^{-4}.

A value of γ greater than 1 suggests that binding the first substrate to the enzyme decreases its affinity for the second substrate. Consequently, the apparent constant K for each substrate A or W increases with an increase in the concentration of the other substrate *(34,35)*.

An E_a value for Lipozyme IM of 60.2 kJ/mol was reported for the hydrolysis of triglycerides in aqueous phase *(32)*. For gas-phase hydrolysis, however, we determined a value for E_a of 24.8 kJ/mol, approx 40% of the value obtained for the aqueous-phase reaction. Yang and Russell *(1)* observed similar results for alcohol dehydrogenase, with $E_a = 7.47$ kJ/mol for the gas-phase reaction, a value 50% lower than the value for the aqueous-phase reaction, $E_a = 14$ kJ/mol.

A detailed analysis of the constant values for γ, K_A, and K_W suggests that there is a preferential route for the reaction. The values for each constant are as follows:

$$K_W = k_{-1}/k_1 = 91 \text{ Pa}$$
$$Km_A = \gamma K_A = k_{-2}/k_2 = 30{,}665 \text{ Pa}$$
$$K_A = k_{-3}/k_3 = 2493 \text{ Pa}$$
$$Km_W = \gamma K_W = k_{-4}/k_4 = 1115 \text{ Pa}$$

in which $k_{1,2,3,4}$ and $k_{-1,-2,-3,-4}$ are the proportionality constants, known as rate constants, and represent reactions in the forward and reverse directions; Km_A is an apparent constant for ester at saturating water; and Km_W is an apparent constant for water at saturating ester, according to Fig. 10. The overall equilibrium constant (*Keq*) between free enzyme (*E*) and *EAW* complex was 2.7×10^6 by either route and, consequently, no rules of thermodynamics were violated.

The value for K_W is approx 30 times lower than that for K_A, suggesting that $k_1 > k_3$ and, consequently, the complex *EW* is formed in the first step. Thus, the overall reaction sequence has a kinetically preferred path, and the steps for this reaction may be represented by an ordered Bi-Bi sequence of steps:

$$E + W \rightarrow EW$$
$$EW + A \rightarrow EWA$$
$$EWA \rightarrow \mathrm{E} + \mathrm{P}$$

The small value of K_W in relation to that of K_A suggests the high affinity of the enzyme for water and that the bond between water and the enzyme results in a change in the enzyme structure that results in the binding of ethyl acetate to the active site. Nevertheless, although this model was in agreement with experimental results, it does not include enzyme denaturation. Consequently, in the future additional Bi-Bi-ordered models with competitive inhibition by both products will be tested.

Conclusion

The results obtained suggest that under restricted water condition the gas-phase hydrolysis of ethyl acetate can be explained by an ordered Bi-Bi sequence of steps. In addition, the affinity of the enzyme for water is high in aqueous-restricted gas-phase reaction, resulting in a preferential route, where water is bound first on the enzyme and subsequently there is a binding of the ester.

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