

KINETIC STUDY OF PALMITIC ACID ESTERIFICATION CATALYZED BY *Rhizopus oryzae* RESTING CELLS

Estudio cinético de la esterificación del ácido palmítico catalizado por células en reposo de *Rhizopus oryzae*

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ABSTRACT

In the present study, a kinetic model for the biocatalytic synthesis of esters using *Rhizopus oryzae* resting cells is proposed. The kinetic study has been made in a range of 30-50 °C and atmospheric pressure. The Influence of operating variables, water content, pH, amount of mycelium was studied. Different values of temperature, initial mycelium concentration and acid/alcohol molar ratio were tested. Initial rates were estimated from the slope of the concentration of palmitic acid, or their corresponding ester at conversions of less than 10%, versus time and reported as mmol l⁻¹ min⁻¹. The values of kinetic constants were computed using the freeware program SIMFIT (<http://www.simfit.man.ac.uk>).

Key words: bound lipase, esterification, fungal resting cells, *Rhizopus oryzae*, palmitic acid, propanol.

RESUMEN

En el presente estudio, un modelo cinético para la síntesis de ésteres usando *Rhizopus oryzae* resting cells es propuesto. El estudio cinético fue realizado en un rango de temperatura de 30-50 °C a presión atmosférica reducida. La influencia de las variables de operación tales como temperatura, pH y contenido de agua fueron estudiadas. Diferentes valores de concentración de micelio y relación molar de ácido/alcohol son ensayadas, Las velocidades iniciales se estimaron de la curva de concentración de ácido palmítico, y su correspondiente conversión a ester en menos del 10%, frente a tiempo y reportadas en mmol l⁻¹ min⁻¹. Los valores de las constantes cinéticas fueron calculados usando el programa freeware SIMFIT (<http://www.simfit.man.ac.uk>).

Palabras clave: Lipasas, esterificación, resting cells, *Rhizopus oryzae*, ácido palmítico, propanol.

INTRODUCTION

Some studies have attempted to understand the functional properties of lipase in processes that involve modification of the properties of fats and oils (Gandhi, 1997). The cost of lipases significantly limits their applicability for bulk production of fuels and chemicals. This prompted research into the potential use of microorganisms such as bacteria, yeast and filamentous fungi that would serve as whole-cell biocatalysts based on their ability of immobilization and the display of functional proteins of interest on their cell surface. Furthermore, simple immobilization techniques and relative ease of process scale up of filamentous fungi renders these particularly practical whole-cell biocatalysts with several commercial advantages (Fukuda *et al.*, 2008). Particularly challenging tasks are those associated with developing rate expressions to characterize several lipase-catalyzed reactions and deciphering the mechanisms involved in the reaction (Paiva *et al.*, 2000).

An important application of enzymatic catalysis in low water organic media is the synthesis of ester catalyzed by lipase (Chulalaksananukul *et al.*, 1999). Kinetic studies using lipases as catalysts in such solvents have only become important in the last few years. The most extended models are based on the application of Michaelis–Menten assumptions. These types of model seem to be valid for the simplest enzymatic reactions. Other authors have proposed a model based on a ping-pong mechanism for the kinetic study of the interesterification reaction. Chulalaksananukul *et al.*, 1999, suggested a model based on the Ping-Pong Bi Bi mechanism for the kinetics of esterification of oleic acid with ethanol using immobilized *Rhizomucor miehei* lipase as biocatalyst. In this sort of reaction, involving two substrates and two products (Bi Bi reaction), the enzyme reacts with the first substrate (the acid in this case) to give a acyl-enzyme intermediate releasing water (the first product). Subsequently, the acyl-enzyme intermediate reacts with the alcohol, as second substrate, releasing the ester as second product (Ping Pong mechanism).

Several other studies confirmed that experimental data for esterification, alcoholysis and ester exchange reactions catalyzed by lipases in various organic solvents were well fitted by this model (Chulalaksananukul *et al.*, 1999; Martinelle and Hult, 1995). In most cases, competitive inhibition by the alcohol was reported. Modeling of lipase-catalyzed reactions in organic solvents has received relatively little attention. In contrast to the wealth of information pertaining to the kinetics of lipases catalyzing hydrolysis reactions, relatively little is reported on the kinetics of esterification and transesterification reactions (Krisha *et al.*, 2001). Most of the models reported to date are based on the application of simple Michaelis-Menten kinetics. Despite the fact that several kinetic studies are reported, the information needed for industrial scale design and analysis continues to be rather limited (Garcia *et al.*, 1999). However, no one has carried out such kinetic studies using resting cells.

The aim of this study was to define for the first time a kinetic model for resting cells as biocatalyst in esterification reactions.

MATERIALS AND METHODS

MATERIALS

The strain of *Rhizopus oryzae* used in this work was isolated as endophytic fungi of *Foeniculum vulgare* (Torres *et al.* 2003). Palmitic acid was purchased from Merck. Propyl palmitate and 1-propanol were obtained from Fluka. Tert-butyl methyl ether (TBME) was purchased from Sigma-Aldrich.

PREPARATION OF RESTING CELLS

A synthetic liquid medium that containing 2 g of asparagine, 1 g of K_2HPO_4 , 0.5 g of $MgSO_4$, 5 mg of thiamine hydrochloride, 1.45 mg of $Fe(NO_3)_3 \cdot 9H_2O$, 0.88 mg of $ZnSO_4 \cdot 7H_2O$, and 0.235 mg of $MnSO_4 \cdot H_2O$ per litre of distilled water was prepared. The initial pH of the medium was adjusted to 6.0. After 250-mL aliquots of the medium were sterilized at 121 °C for 15 min, 1% (v/v) of refined sunflower oil was added aseptically. The medium was inoculated with 2.5 mL of an *R. oryzae* spore suspension ($1-4 \times 10^6$ spores/mL) and then incubated at 28 °C for 5 d using an orbital shaker at 200 rpm. Mycelia were harvested from the culture medium using a Buchner funnel, and washed with distilled water and acetone successively. The mycelium was then dried under vacuum for 18 h and ground to a powder (Torres *et al.* 2000).

GENERAL METHODS

All reactions were carried out for triplicate in screw cap vials with a Teflon-faced rubber liner under the pressure generated by the system. The vials were heated a 40 °C using an aluminium block, an agitation rate of 200 rpm, TBME as solvent and 10 µL of tridecane was added as an internal standard.

DETERMINATION OF KINETICS CONSTANTS

Initial rate were determined from the time course of propyl palmitate synthesis using curve fitting by regression analysis and determining the initial slope of the tangent to the curve. Low conversions (<10%) were used to minimize inhibition by products (propyl palmitate and water).

The effect of both the alcohol and the acid on the reaction rate were investigated by esterifying various fixed initial quantities of palmitic acid with various initial concentrations of propanol and vice versa. Resting cells of *Rhizopus oryzae* concentration of 6 g/L was used in all the experiments.

The initial reaction rates obtained at various acid and alcohol concentrations were fitted to Michaelis-Menten kinetics with Ping-Pong Bi-Bi mechanism by non-linear regression using freeware program SIMFIT (<http://www.simfit.man.ac.uk>).

EFFECT OF TEMPERATURE ON LIPASE ACTIVITY

Three vials containing 30 mg of lyophilized mycelium suspended in 0.1 mL of MTBE were prepared for each reaction time (0, 5, 20 and 40 min) and corresponding temperature (30, 35, 40, 45 and 50 °C). Each vial was preincubated at the appropriate temperature for 30 min, with a magnetic stirrer turning at 200 rpm. Then, 0.5 mL of a solution of 125 mM palmitic acid and 125 mM propanol in MTBE and 10 mL of tridecane as

internal standard, warmed to the same temperature, were added to each vial. The reaction mixtures were then stirred, with a magnetic stirrer turning at a speed of 200 rpm, and 100 μ L aliquots were withdrawn at various reaction times and filtered. The amount of propyl palmitate was quantified by gas chromatography, as described below. For the temperature dependency Activation Energy were calculated using the Arrhenius equation (Riet and Tramper, 1991).

$$K_{n,T} = k_{n,T_{ref}} \cdot e^{(-\Delta H^*/R \cdot (1/T - 1/T_{ref}))}$$

In this equation $K_{n,T}$ is the reaction-rate constant at temperature T, while $k_{n,T_{ref}}$ is the reaction-rate constant at reference temperature (T_{ref}) 293 K and ΔH^* is the activation- enthalpy change.

EFFECT OF pH ON LIPASE ACTIVITY

A potassium hydrogen phthalate ($\text{KHC}_8\text{H}_4\text{O}_4$) buffer was used to control pH in the 3-5.5 range. A potassium dihydrogenphosphate (KH_2PO_4) buffer was used to control pH in the 6-7.5 range. Finally, a tris(hydroxymethyl)- aminomethane ($\text{C}_4\text{H}_{11}\text{NO}_3$) buffer was used to control pH at 8. All the buffers were made up from stock solutions of 0.05 M salts using either 0.1 M NaOH or 0.1 M HCl to reach the required pH. The mycelium was first incubated in buffered solutions for 3 h at 26 °C. Then, it was filtered, washed three times with distilled water and three times with acetone, lyophilized for 2 h, and grounded to a powder.

All assays were carried out as described above. Samples were taken at 0- 30 -60- 90 and 120 s intervals and the ester presence was analyzed by gas chromatography.

EFFECT OF WATER CONTENT

Lyophilized mycelia were dried under vacuum over P_2O_5 at room temperature for 2h (Chamorro *et al.*, 2001). Reactions were carried out immediately after drying to prevent the mycelium from taking up water. All assays were carried out as described above modifying the water content (1 to 4 % v/v) .

EFFECT OF THE AMOUNT OF *R. ORYZAE*

Experiments were conducted to identify the influence of the amount of biocatalyst on the initial reaction rate. Variable amounts of mycelium (20-40-60-90-120 mg/mL) and 5 μ L of water were added a different vials containing the mixture of reaction. All assays were carried out as described above.

ANALYSIS AND CHARACTERIZATION

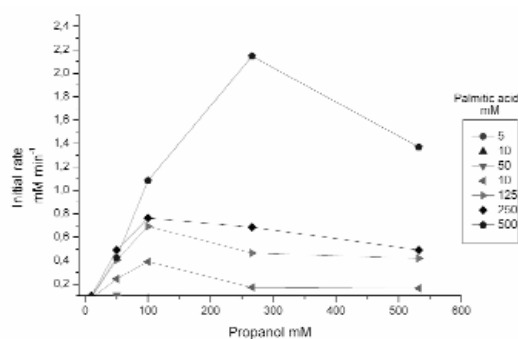
Aliquots of the reaction mixture were analyzed for GC with a Fisons series Chromatograph equipped with an FID detector and capillary column (30 m x 0.25 mm diameter, 0.25 μ m film thickness), unless otherwise noted. Hydrogen (0.5 mL/min) was used as carrier gas. $T_{injector} = 250$ °C, $T_{detector} = 300$ °C, $T_{column} = 110$ -250 °C (10 °C/min), and 250 °C (13 min). The chromatographic peaks were recorded and integrated using the Millenium 32 computer software package (Waters Cromatografia SA, Spain). All the samples were analyzed by triplicate.

RESULTS AND DISCUSSION

REACTION KINETIC

The reaction kinetics using *Rhizopus oryzae* resting cells were investigated by studying the effect of the concentration of both substrates (propanol and palmitic acid) on the initial esterification rate. Multiple combinations of propanol-palmitic acid concentration were fitting considering a factorial design methodology (Cobb, 1998). A graphical representation of the initial rates against propanol (or palmitic acid) concentration at several fixed values of palmitic acid (or propanol) concentration, was used. The effect of the concentration of both palmitic acid and 1-propanol on the initial rate of the reaction was then studied.

The initial rate increases with the increase in the propanol concentration until a determined concentration than initial rate decreases, showing a possible propanol inhibitory effect (Fig. 1). The effect of palmitic acid on the initial esterification rate, at fixed propanol concentrations, shows a similar behavior (Fig. 2). At high palmitic acid concentrations an inhibitory effect was also observed, that could be originated by the increase of the acid substrate and/or to mass transfer diffusional limitations (Oliveira *et al.*, 2001). Several authors have reported on alcohol inhibition during lipase-catalyzed esterification reactions. (Dumont *et al.*, 1992; Gillies *et al.*, 1987) indicated the presence of excess ethanol drastically effect the yields of the ethyl butyrate and other ethyl ester, while butyric acid did not showed any inhibitory effect. Recently, Duan *et al.*, 1997, reported the inhibition of *Candida Antarctica* lipase by propanol. Butanol was also shown to exert inhibitory effects on the synthesis of butyl laurate (Gandhi *et al.*, 1995). In contrast to this observation, butanol has been reported to have no inhibitory effects on the synthesis of butyl butyrate (Gillies *et al.*,



1987).

Figure 1. Effect of initial propanol concentration on the reaction rate at various initial acid concentrations.

The double reciprocal plot of the initial reaction rate versus the reciprocal palmitic acid concentration at several propanol concentrations is shown in figure 3 and figure 4. A set of nearly parallel lines was obtained indicating a likely Ping-Pong Bi-Bi mechanism. In this typical reaction sequence, the enzyme initially forms a noncovalent enzyme-palmitic

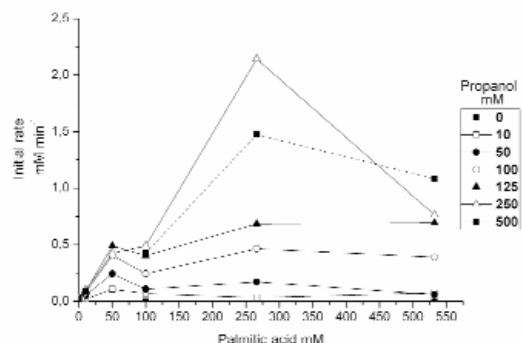


Figure 2. Effect of initial palmitic acid concentration on the reaction rate at various initial propanol concentrations.

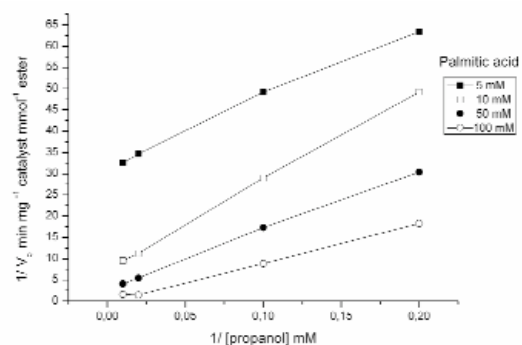


Figure 3. Double-reciprocal plot of the initial esterification rate varying palmitic acid concentrations.

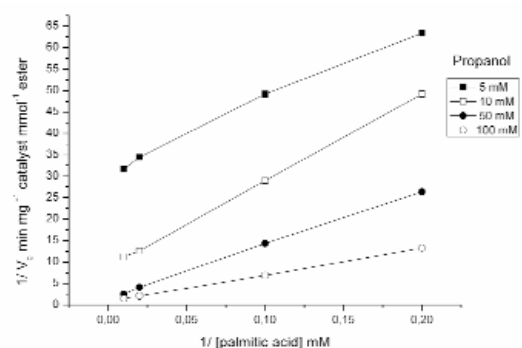


Figure 4. Double-reciprocal plot of the initial esterification rate at varying propanol concentrations.

acid complex, which then subsequently transforms by a unimolecular isomerization reaction to acyl-enzyme intermediate, with the concomitant release of water. Then, the modified enzyme reacts with 1-propanol to form the modified enzyme-propanol complex, which is also isomerized by a unimolecular reaction to a enzyme palmityl-propyl ester complex, which then yields the product palmityl-propyl ester, and free enzyme (Fig. 5). A similar mechanism has been proposed for lipase-catalyzed esterifications in other nonconventional media, such as esterification of oleic acid and

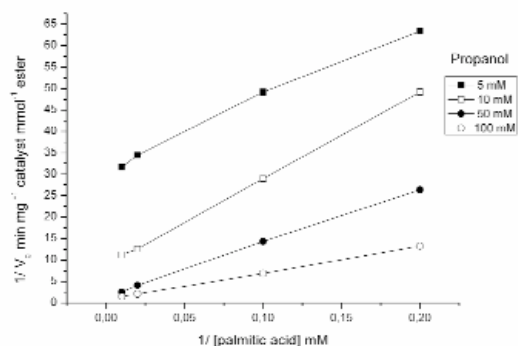


Figure 5. Ternary complex mechanism.

ethanol in n-hexane (Chulalaksanakul *et al.*, 1990) and esterification of lauric acid and menthol (Stamatis *et al.*, 1992).

The kinetic parameters can be obtained according to the following equation:

$$1/V_o = [1 + K_{m(PA)} / [PA] + K_{m(P)} / [P]] 1/V_{max}$$

Where $K_{m(PA)}$ and $K_{m(P)}$ are the Michaelis-Menten constants with respect to palmitic acid and 1-propanol respectively, V_{max} is the maximum esterification reaction rate, and $[PA]$ and $[P]$ represent the initial concentrations of palmitic acid and 1-propanol. The values for the model parameters that were determined from the graph were: $K_{m(PA)} = 99.8 \text{ mmol l}^{-1}$, $K_{m(P)} = 331.35 \text{ mmol l}^{-1}$ and $V_{max} = 2.14 \text{ } \mu\text{mol min}^{-1} \text{ mg}^{-1} \text{ catalyst}$. The values of K_m showed one affinity between palmitic acid and the free enzyme. The poor information using resting cells did not allow confronting results. However, similar assays have been carried out utilizing supported enzymes based on the assumption of a ping pong mechanism. Oba *et al.*, 1994, report rate constants for hydrolysis reactions using immobilized *R. oryzae* lipases; Krishna *et al.*, 2001 present kinetic study for lipase-catalyzed esterification of butyric acid and isoamyl alcohol, the values of the apparent kinetics parameters were computed as: $V_{max} = 11.72 \text{ } \mu\text{mol min}^{-1} \text{ mg}^{-1}$; $K_m \text{ acid} = 3.03 \text{ mmol l}^{-1}$; $K_m \text{ alcohol} = 3.06 \text{ mmol l}^{-1}$. Besides a determination of the kinetics parameters the effect of water presence, enzyme concentration, pH and reaction temperature on the initial reaction rate (V_o) were also studied.

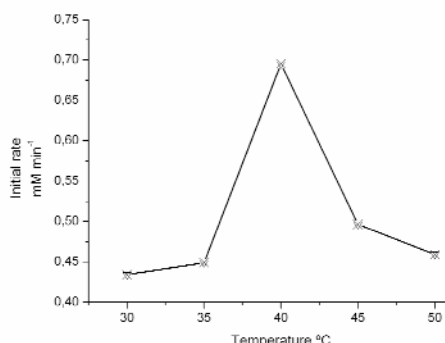
EFFECT OF TEMPERATURE ON CATALYTIC RATE

Figure 6 shows the effect of temperature on the initial rate reaction. It shows initially as the temperature increased the reaction rate increased. This could be due to the increase in rate constant with temperature and partly due to increase in interfacial area. However, the initial rate decreased sharply after 50 °C, which was due to the deactivation of the enzyme. It is known that most proteins tend to decompose at temperatures above 50 °C (Bailey and Ollis, 1986). The most common cause for the inactivation of enzymes at elevated temperature is the loss of the native, catalytically competent conformation, i.e. thermodenaturation. The work of Turner *et al.* (Turner *et al.*, 1995) demonstrated that the temperature at which a protein undergoes thermal

denaturation (T_d) is strongly dependent on the amount of water associated with the protein.

In similar study, Razak, *et al.*, 1999, found that the optimal temperature for activity of the membrane-bound lipase obtained from a *R. oryzae* was 37 °C.

The experimental results showed in figure 6 were used to determine the effect of temperature on the rate constant, K_{cat} . The activation energy was obtained using the Arrhenius equation from all reaction-rate constants showing temperature dependency. The activation energy of 5.6 Kcal mol⁻¹ calculated is lower than the reported activation energies for most enzyme reactions. Thus, Kim and Chung, 1989, reported a value of 7.0 Kcal mol⁻¹ for the hydrolysis of palm kernel oil in reversed micelle system employed lipases of *Rhizopus arrhizus*. Nevertheless, it is necessary to



have in mind that no one of the results reported nowadays are achieved using fungal resting-cells but either free or immobilized enzymes (Bailey and Ollis, 1986).

Figure 6. Effect of temperature on the initial rate.

EFFECT OF WATER CONTENT ON CATALYTIC RATE

In figure 7 the effect of water content on the catalytic rate is shown. The initial velocity of esterification decreases when water content increases. For higher water content (4 g/100 g mycelium), the inhibitory effect of water is more remarkable. This inhibition could be worthy of the competition between water a propanol for the acyl-enzyme intermediate. It is worthy known that water plays a crucial role on enzyme activity. Once the nature of the biocatalyst and the nature of the solvent are fixed, the water sorption isotherm depends on the amount of added water (Chamorro *et al.*, 2001). A minimal amount of water is necessary for the enzyme to ensure its optimal conformation and the to become optimally active (Zaks and Klivanov, 1988; Monot *et al.*, 1991). However, an excess of water decreases the enzyme catalytic activity both from kinetic and thermodynamic perspectives (Chulalaksananukul *et al.*, 1990; Marty *et al.*, 1992). The presence of excess water favors hydrolysis over synthesis reactions (Rocha *et al.*, 1998). Water content may therefore affect reaction rate, yield, and the stability of enzymes (Kaur *et al.*, 1997). Thus, water content must generally be strictly controlled for ester synthesis (Boutur *et al.*, 1995). Moreover, the water content requirements for biocatalysis are highly dependent on the solvent (Halling, 1997), with highly polar

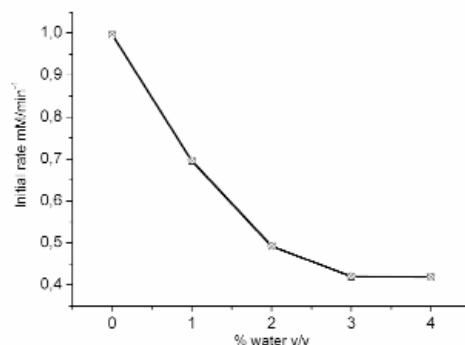
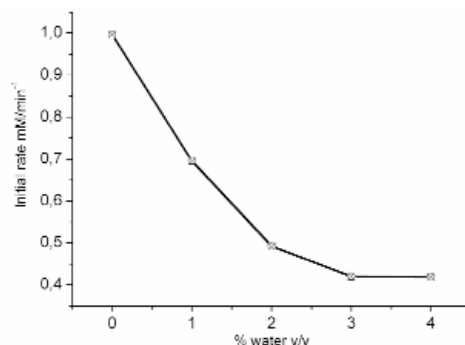


Figure 7. Effect of water on the initial reaction rate.

solvents tending to strip essential water from the enzyme (Mensah *et al.*, 1998).

EFFECT OF INITIAL AMOUNT OF *R.ORYZAE* ON CATALYTIC RATE

The effect of increasing mycelium amount on the initial rate of reaction is shown in figure 8. The initial rate of reaction increased linearly with enzyme concentration. However in assay made by Albasi *et al.*, 1999, for the hydrolysis of sunflower oil, they observed that at high enzyme concentrations, this increase tends to fall in the yields of esterification. This phenomenon is explained by hypothesizing that at high concentrations of enzyme the interfacial area is totally saturated with substrate.



Considering that the increase of the content of *R. oryzae* resting cells led to a linear increase in V_0 , it is worthy to accept that this enzymatic reaction is kinetically controlled.
Figure 8. Effect of initial concentration of *Rhizopus oryzae* resting cells on the initial rate.

EFFECT OF PH ON CATALYTIC RATE

Figure 9 shows the variation in the initial rate between pH 3 and pH 8. The highest initial rate was observed at pH 7 observing a sharp decrease at higher pH. These results agree with the already describer for the synthesis of propyl linoleate using the same biocatalyst (Torres *et al.*, 2003). Whereas this biocatalyst shows activity in both acidic and neutral conditions, several authors have reported that lipases are activity only in acidic con-

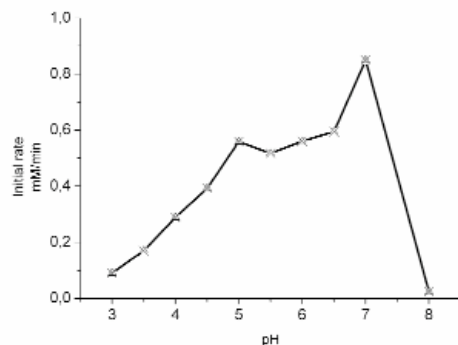


Figure 9. Effect of pH on the initial reaction rate.

ditions. Such lipases include those from *R. rhizodiformis* (pH 6.0; Razak *et al.*, 1997), *R. delemar* (pH 5.6) and *R. oryzae* (pH 7.0; Razak *et al.*, 1999).

CONCLUSIONS

The kinetics corresponding to the esterification of palmitic acid and propanol in MTBE catalyzed by *Rhizopus oryzae* resting cells are suggested to agree with a Ping Pong Bi Bi mechanism. Whereas the addition of water, produce a loss of the enzymatic activity, the optimal pH and temperature were pH 7 and 40 °C respectively. The estimated rate constants show an Arrhenius dependence on temperature until inhibitory effects are observed. The activation energy calculated is 5.6 Kcal mol⁻¹. Other constants are : Vmax = 2.14 mmolmin⁻¹mg⁻¹, Km_{propanol} = 158 mmol l⁻¹, Km_{acid} = 97.07 mmol l⁻¹, U = 72 µmol.min.mg.

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