Appl Biochem Biotechnol (2008) 144:213–223
DOI 10.1007/s12010-007-8114-y

# Effect of Chain Length on Enzymatic Hydrolysis of *p*-Nitrophenyl Esters in Supercritical Carbon Dioxide

**Mahesh N. Varma · Giridhar Madras**

Received: 6 June 2007 / Accepted: 21 November 2007 /
Published online: 25 January 2008


**Abstract** The effect of chain length on the enzymatic hydrolysis of various *p*-nitrophenyl esters was investigated. Specifically, the hydrolysis of various esters *p*-nitrophenyl butyrate (PNPB), *p*-nitrophenyl caprylate (PNPC), *p*-nitrophenyl laurate (PNPL), *p*-nitrophenyl myristate (PNPM) and *p*-nitrophenyl palmitate (PNPP) was studied in supercritical carbon dioxide ($ScCO_2$) with lipase (Novozym 435). This indicates that the conversion of nitrophenyl esters decreases with increasing chain length. The effect of various parameters such as amount of water added, temperature, and enzyme loading was studied. The optimum temperature for the hydrolysis of PNPB and PNPC was 50°C but was 55°C for PNPL, PNPM, and PNPP in $ScCO_2$. The reactions were also conducted in acetonitrile as the solvent, and it was found that the reactions reach equilibrium much faster in $ScCO_2$ than in acetonitrile. The kinetics of the hydrolysis reactions were modeled using a Ping Pong Bi Bi model.

**Keywords** Hydrolysis · PNP · PNPL · Supercritical carbon dioxide · Equilibrium

## Introduction

Several enzymatic reactions have been studied in organic solvents [1–12], supercritical fluids [12–19], and in gas phase [20–21]. In most of enzymatic reactions such as esterification, transesterification, and hydrolysis, lipases (triacyl glycerol hydrolase's, EC 3.1.1.3) are used. These enzymes are hydrophobic in nature, and reactions are heterogeneous. Thus, reactions occur at the interfacial area between enzyme and solvent and are diffusion-controlled. Supercritical fluids are fluids above the critical temperature and pressure with liquid-like density and solubility and gas-like diffusivity. These properties of supercritical fluids favor heterogeneous enzymatic reactions and thus can be used as an alternative for organic solvents. Lipases are found to be active and stable in supercritical fluids [18], whereas some enzymes are even reported to increase in stability with an increase in pressure [22]. As the solubility is significantly influenced by pressure and

M. N. Varma · G. Madras (✉)
Department of Chemical Engineering, Indian Institute of Science, Bangalore 560012, India
e-mail: giridhar@chemeng.iisc.ernet.in

temperature, separation can be achieved in a single step during downstream processing. Among all supercritical fluids, supercritical carbon dioxide ($ScCO_2$) is cheap, non-flammable, non-toxic, with a near-ambient critical temperature (31.1 C), and a moderate critical pressure (73.8 bar). The hydrolysis of PNPE has been studied [23–27] extensively primarily to measure the activity of lipase [28–29]. Hui et al. [7] studied the hydrolysis of esters of *p*-nitrophenol bound by amylase in the $Me_2SO$–$H_2O$ (dimethyl sulfoxide and water) system. Kodaka [24] investigated the hydrolysis of *p*-nitrophenyl acetate, *p*-nitrophenyl hexanoate, and *p*-nitrophenyl laurate (PNPL) with various catalysts. Tsutomu and Janos [25] described the rate enhancement of the base hydrolysis of PNPL in presence of surfactant and polymerized surfactant.

However, there are no studies on the effect of chain length on the hydrolysis of PNPE in organic media or $ScCO_2$ using Novozym 435. Further, studies on the kinetics of the hydrolysis of PNPE are lacking. Therefore, the objective of this work was to study the effect of chain length of PNPE and the influence of various parameters such as temperature, time, and water content for the enzymatic hydrolysis with Novozym 435 lipase in organic solvent (acetonitrile) and in $ScCO_2$. The kinetics of the reactions were also determined and modeled using Ping Pong Bi Bi mechanism.

## Experimental

*Materials* All *p*-nitrophenyl esters (PNPE), *p*-nitrophenyl butyrate [$C_{10}$] (PNPB; 99%), *p*-nitrophenyl caprylate [$C_{14}$] (PNPC; 97%), *p*-nitrophenyl laurate [$C_{18}$] (PNPL; 98%), *p*-nitrophenyl myristate [$C_{20}$] (PNPM; 97%), *p*-nitrophenyl palmitate [$C_{22}$] (PNPP; 98%), were purchased from Fluka Chemie AG, Switzerland. Novo Nordisk A/S, Denmark generously donated Novozym 435. Acetonitrile (99%, S.D. Fine chemicals, Mumbai), hydrochloric acid (35%), hydroxymethyl (Tris) amino methane (99.8%), and silica gel (Merck, India), $CO_2$ (99%, Vinayaka Gases) were used.

*Experiments* All reactions are carried out at a substrate (PNPE) concentration of $2\times10^{-4}$ mol $l^{-1}$. Reactions in acetonitrile solution were performed in 4 $cm^3$ capped glass vials. About 3.5 $cm^3$ reacting mixture of PNPE with required concentration were taken in vials and incubated at desired temperature for 2 min. The required amount of enzyme was then added to vials, and the mixture was then incubated for a desired interval of time. The reacted mixture was then quenched in an ice bath.

The reactions in $ScCO_2$ were performed in 7 $cm^3$ tubular batch reactors. The reactors were loaded with reactants and enzymes. The same substrate (PNPE) concentration (as that in case of reaction in acetonitrile) of $2\times10^{-4}$ mol $l^{-1}$ was used. As it was very difficult to load the reactants in such small quantity, a 25 μl of PNPE solution in acetonitrile [26] with required concentration was loaded in reactor. The reactor was pressurized to an initial pressure of 70 kg $cm^{-2}$ at room temperature as that in gas cylinder, where in the density of carbon dioxide is 790 kg $m^{-3}$ at the initial condition. The pressurized reactor was then immersed in water bath maintained at the desired constant temperature (±0.5 °C) and constant pressure (±1 kg $cm^{-2}$). Depending on the temperature, the pressure of reactor increased to 75–100 kg $cm^{-2}$, but the density is constant.

*Sample Analysis* In case of reactions in acetonitrile, 0.25 ml of clear supernatant liquid from the vials was added to 3.0 ml of 0.1 mol $l^{-1}$ HCl–Tris buffer solution of 9.0 pH. In case of reactions in $ScCO_2$, the reactor was quenched and depressurized, and the mixture in

reactor was eluted with the buffer solution. The enzyme was settled by centrifugation at 4,000 rpm for 2 min. The absorbance of the resulting solution was measured against buffer at 410 nm in a UV-Vis spectrophotometer (Shimadzu UV 2100), and the concentration of PNP was determined from the calibration curves. Some experiments were repeated to check the reproducibility of the results, and conversions were found to be within 2%.

## Result and Discussion

*Effect of Water Content* Whereas water is essential for hydrolysis and for maintaining enzyme activity, excess water may reduce the reaction rate by forming an aqueous layer around the enzyme and contributing to mass transfer resistance. The water content of acetonitrile used is 0.11 mol $l^{-1}$, which is higher than the stoichiometric water requirement ($2\times10^{-4}$ mol $l^{-1}$). Because the solvent is in liquid phase, no significant change in mass transfer resistance will occur with addition of water. Therefore, the effect of water addition was not studied for reactions in acetonitrile. For $ScCO_2$, the effect of water addition was investigated at 50°C and 3 mg of enzyme loading for 5 h. Figure 1 shows the effect of water addition during the hydrolysis of PNPE. Whereas a conversion of only about 5% is obtained in dry $CO_2$, a sharp increase in conversion is obtained with water addition up to 8 μl, which remains constant until 90 μl, but decreases with addition of more water before attaining an equilibrium value. Although the stoichiometric requirement of water for the reaction is very low ($2\times10^{-4}$ mol $l^{-1}$), the water present in the enzyme is either very low for reaction or total water in enzyme cannot be used for reaction. Therefore, there is a sharp increase in conversion with an initial addition of water. However, excess water does not influence conversion, and the plot of conversion with water attains a plateau. The solubility of water in $ScCO_2$ is very low [30–31] (0.35 mol % at temperature 50°C and pressure 78 kg $cm^{-2}$) and corresponds to about 7 μl for this system. The conversion of PNPE decreases when the water added is in excess of solubility leading to formation of a layer around enzyme and reduction of rate. For low water addition, the conversions of the all nitrophenyl esters are similar. At a higher water concentration, however, the conversion of PNPB is the highest, and the conversion decreases with increases in chain length leading to the lowest conversion for PNPP.

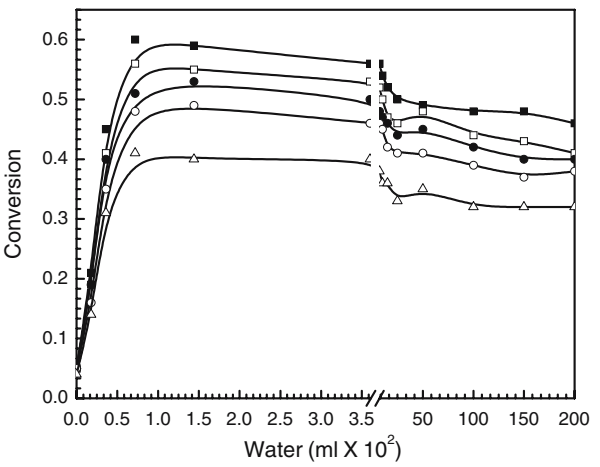


**Fig. 1** Effect of water addition on conversion during hydrolysis of PNPE in $ScCO_2$ for 5 h at 50 °C and 3 mg enzyme. PNPB (*filled square*), PNPC (*open square*), PNPL (*filled circle*), PMPM (*open circle*), PNPP (*open triangle*)

Thus, it is clear that water plays an important role on the rate of reaction. Whereas a dry enzyme exhibits no activity, an excess of water adsorbed on the enzyme can lead to irreversible denaturation [32]. The dependence of enzyme activity on water content in supercritical $CO_2$ can be represented as a bell-shaped curve with a plateau corresponding to the optimum moisture. The optimum water content in Lipozyme was between 8 and 12% (g $g^{-1}$ of supported enzyme) [33], whereas it was less in other studies. The effect of water content is a crucial parameter in determining the conversion and has been widely investigated [34–38]. Thus, the comparison with literature indicates that our experiments were performed within the range of optimum water content in carbon dioxide. This is consistent with an earlier observation of an optimum of 44 mM for an immobilized enzyme [39]. Thus, the decrease of conversions on the addition of water may be attributed to the hydrophilic hindrance for the hydrophobic substrate to react with the enzyme [40] or deactivation of the enzyme by water.

## Effect of Temperature

The effect of temperature was investigated with 3 mg enzyme loading, 5 h in $ScCO_2$ and 24 h for acetonitrile. The conversion initially increases and then decreases, similar to that observed in previous studies [15, 19]. Figure 2a and b shows the optimum temperature is 50°C for all reactions in acetonitrile. However, the optimum temperature was 50°C for the synthesis of PNPB and PNPC and 55°C for the synthesis of PNPL, PNPM, and PNPP in $ScCO_2$. The yield decreased at temperatures below and above to this value. Temperature influences the three dimensional conformation and hence the stability of the enzyme. On the other hand, it affects the partition of substrates between the $ScCO_2$ phase and enzyme phase. In $ScCO_2$, increase in temperature leads to the increase in substrate solubility [41] and decrease in density and viscosity of $ScCO_2$ system and, apparently, is favorable for the mass transfer rate of substrates and products in the reaction system. However, increase in reaction temperature adversely affects enzyme stability. It has been pointed out [42] that high temperature might lead to changes in enzyme conformation and free energy of the reaction system and, thus, might affect enzyme-substrate binding capacity and the yield of product. In addition, temperature also affects the partition of substrates between the $ScCO_2$ phase and the enzyme phase [41]. The optimum temperature observed in this study is similar to optimum temperature of 40–60 °C for synthesis of isoamyl acetate [42], isoamyl butyrate [19], ethyl palmitate [17], lavandulyl acetate [43], ethyl myristate [34], and geranyl acetate [10] in $ScCO_2$. The conversion of PNPB was similar in both $ScCO_2$ and acetonitrile. However, the conversion of PNPC was considerably higher in $ScCO_2$, whereas the conversion of PNPL, PNPM, and PNPP was higher in acetonitrile.

## Effect of Enzyme Loading

From the economical point of view, achieving high reaction rates utilizing low enzyme concentration is important. Therefore, the impact of enzyme loading on initial reaction rate was studied. The effect of enzyme loading was investigated at 50 °C for 5 and 24 h in $ScCO_2$ and acetonitrile, respectively. Figure 3 shows that the conversion increases with enzyme loading up to 3 mg. At a loading of 3 mg enzyme or less, the conversion of PNPB and PNPL are the same in acetonitrile (Fig. 3a). However, with increasing enzyme loading, PNPB gives higher conversion when compared to PNPL. In acetonitrile, the conversion decreases with increasing chain length expect for PNPC. In $ScCO_2$ (Fig. 3b), the conversion

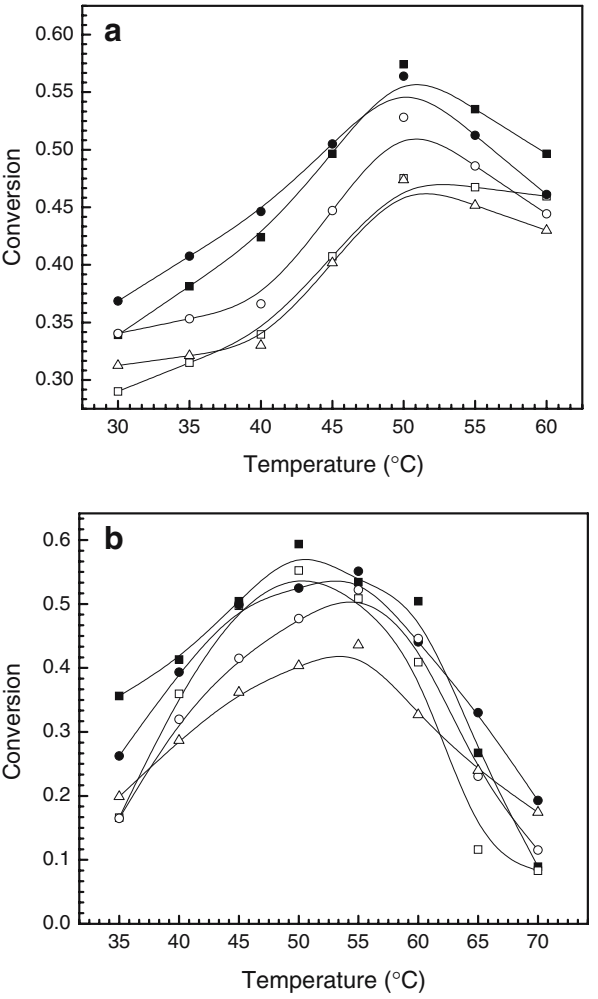


**Fig. 2** Effect of temperature on conversion during hydrolysis of PNPE in **a** acetonitrile for 24 h and **b** in $ScCO_2$ with 3 mg enzyme for 5 h. See Fig. 1 for legends

decreases with increasing chain length for all nitrophenyl esters. A further increase in enzyme loading than 3 mg does not affect the overall conversion, similar to the results obtained in previous work for the synthesis of flavors in $ScCO_2$ [19]. This quantity was slightly lower than that reported by Gubicza et al. [44] in organic media and similar to the value reported for the synthesis of isoamyl acetate in free-solvent media [45] in supercritical carbon dioxide [42]. The optimum amount of enzyme is also similar to that observed for esterification and transesterification synthesis of tetrahydrofurfuryl butyrate [4] in heptane, esterification *n*-butyl acetoacetate [46] in toluene, and for the synthesis of isoamyl acetate [19], isoamyl butyrate [19], and ethyl palmitate [17] in supercritical carbon dioxide.

## Kinetics of Reactions

The kinetics of reaction was studied with 3 mg enzyme at 50 °C. Figure 4a shows the variation of conversion with time for reactions in acetonitrile. The conversions of all

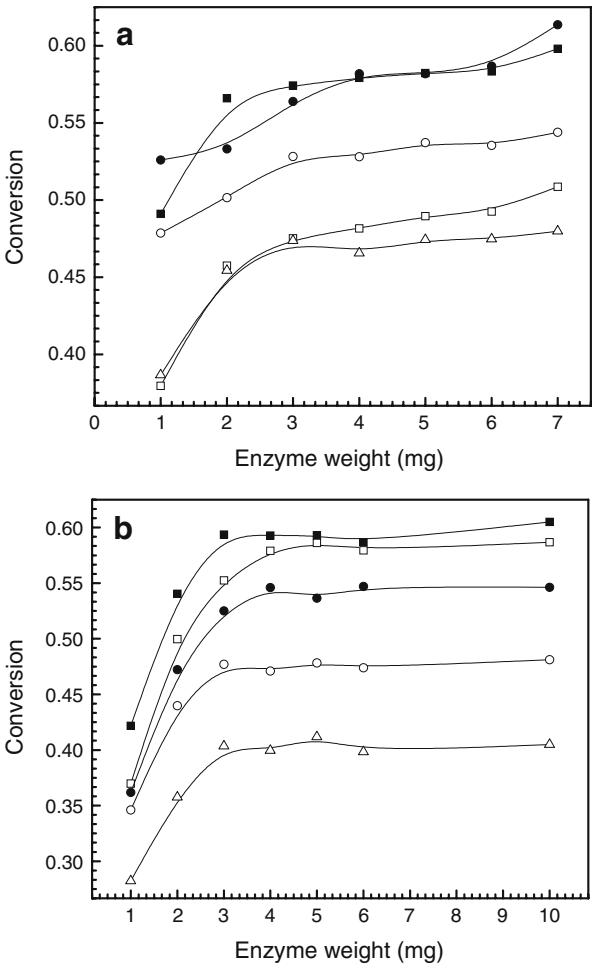


**Fig. 3** Effect of enzyme loading on conversion during hydrolysis of PNPE in **a** acetonitrile for 24 h, **b** $ScCO_2$ for 5 h at 50 °C. See Fig. 1 for legends

nitrophenyl esters were similar for the first 3 h, but the conversions were different at longer times. As in the previous cases, the conversion of nitrophenyl esters decrease with increasing chain length except for PNPC, whereas in $ScCO_2$, the conversion decreases with increasing chain length for all nitrophenyl esters. All the reactions took around 24 h to achieve equilibrium with a maximum of 60% conversion for PNPB. The initial rate of reaction is high with a sharp increase in conversion to about 50% of the equilibrium in the first 4 h, and the reaction rate slows to reach the equilibrium conversion in around 24 h in acetonitrile. An equilibrium conversion of 58.5% is obtained for PNPL, which is much higher than that obtained in aqueous medium [17]. This indicates that the liquid plays an important role in deciding film resistance around enzyme. As this liquid layer is not present in case of $ScCO_2$, the reaction in $ScCO_2$ is faster (Fig. 4b), and thus equilibrium is reached in less than 3 h. Thus, the kinetics of the reaction is much faster in $ScCO_2$ compared to that in acetonitrile. The equilibrium conversion of PNPL is 52% with this enzyme, which is greater than those obtained with hog pancreas lipase (22.5%) and *Penicillium roqueforti* lipase (15.3%) in $ScCO_2$ [15] indicating the efficacy of Novozym 435 for this reaction.

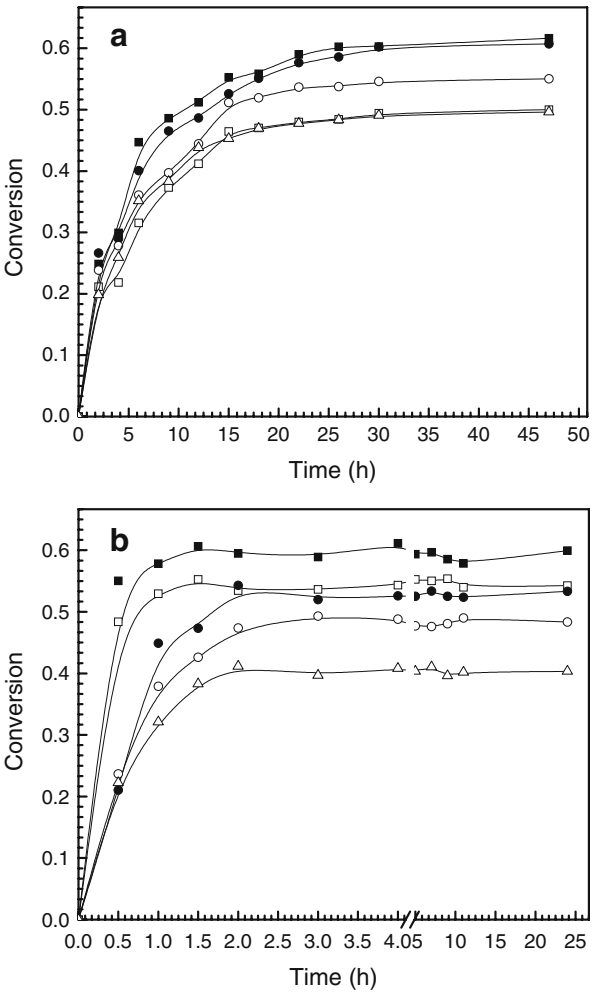


**Fig. 4** Kinetics of hydrolysis reactions of PNPE in **a** acetonitrile, **b** $ScCO_2$ with 3 mg enzyme at 50 °C. See Fig. 1 for legends

Chulalaksananukul et al. [47] first proposed 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 type of reaction, involving two substrates and two products (Bi Bi reaction), the enzyme reacts with the first substrate to give a covalently modified enzyme intermediate and then releases the second product (Ping Pong mechanism). Several other studies confirmed that experimental data for hydrolysis, esterification, alcoholysis, and ester exchange reactions catalyzed by lipases in various organic solvents were well fitted by this model [48–54]. In most cases, competitive inhibition by the alcohol was reported. Several studies in supercritical carbon dioxide also showed that experimental data were in good agreement with this model [55–57]. Some authors have demonstrated in both conventional and in supercritical media that the kinetic mechanism for these reactions was a Ping Pong Bi–Bi with competitive alcohol inhibition [58–60]. The Ping pong bi bi mechanism has thus been extensively used for lipase catalyzed esterification [61] and transesterification [62] in organic solvents and in $ScCO_2$ [61, 63].

In this mechanism, the equation for the initial velocity of reaction, $V_i$, in terms of the maximum velocity, $V_{max}$, is

$$V_i = \frac{V_{max} C_e C_w}{C_e C_w + K_w C_e \left[1 + \frac{C_e}{K_{ie}}\right] + K_e C_w \left[1 + \frac{C_w}{K_{iw}}\right]} \quad (1)$$

In Eq. 1, $C_e$ and $C_w$ represent the initial molar concentration of ester and water; $K_e$ and $K_w$ represent the apparent Michaelis constant and $K_{ie}$, $K_{iw}$ the apparent inhibition constant for corresponding ester and water, respectively.

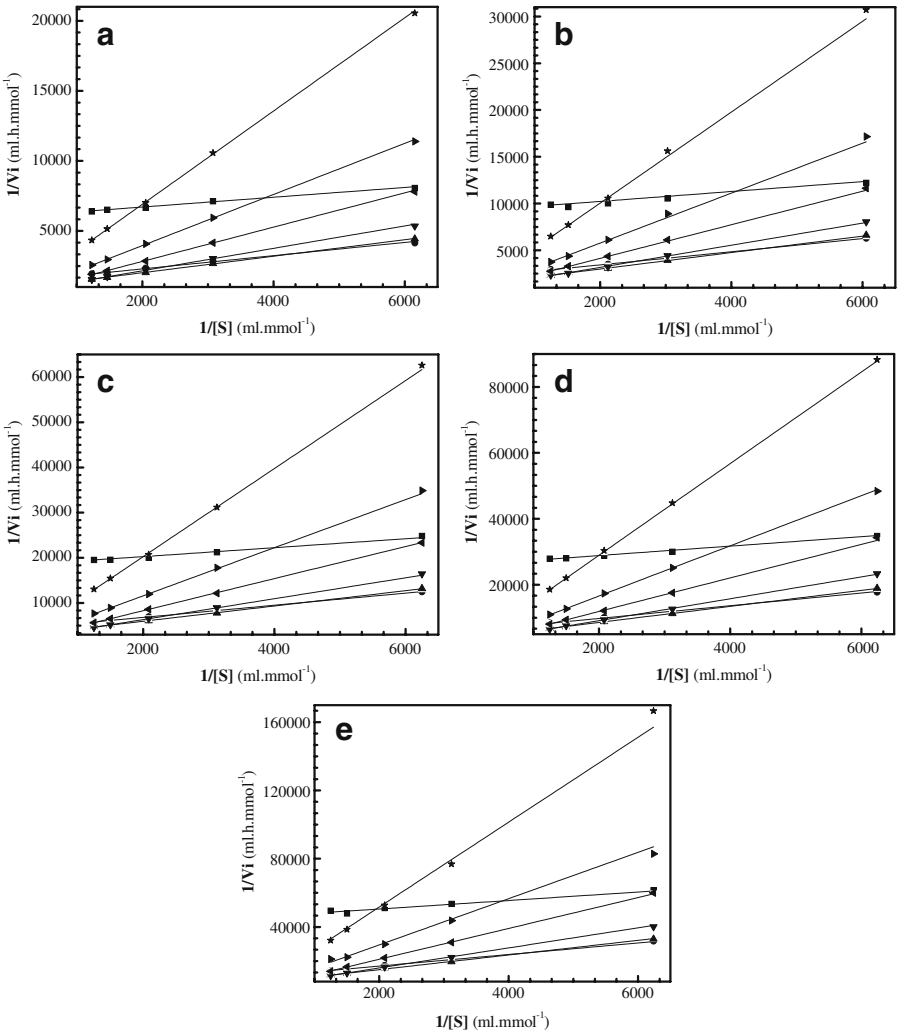


**Fig. 5** Lineweaver and Burk representation of the variation of the initial velocity of the reaction, $V_i$, with the initial molar concentration of ester, [**S**], for the hydrolysis of **a** PNPB, **b** PNPC, **c** PNPL, **d** PNPM, **e** PNPP with water in presence of 10 mg enzyme at 50 °C. The *lines* represent the model prediction. Legends represent water concentrations (in mol/l) of 0.04 (*square*), 0.20 (*circle*), 0.36 (*triangle*), 0.63 (*inverted triangle*), 1.19 (*left arrowhead*), 1.98 (*right arrowhead*), 3.97 (*star*)

The kinetic parameters were determined by regressing the experimental data with the model prediction. The Lineweaver and Burk plot shows the comparison of the experimental data and the model (Figs. 5a–e). The kinetic parameters (Table 1) indicate that the ester does not show any inhibition, whereas water has inhibitory effect on the reaction. The initial velocity is fastest for the hydrolysis of lower chain length ester (PNPB) and decreases with increasing chain length.

In summary, this study has investigated the effect of various parameters like temperature, enzyme loading, and water addition on the enzymatic hydrolysis of various PNPEs in supercritical carbon dioxide and in acetonitrile. The kinetics of the reactions were also determined. The key determination of the study that the equilibrium conversion decreased with increasing chain length of the ester. Thus, the highest conversion was obtained for PNPB, whereas the lowest conversion was obtained for PNPP. It is interesting, however, that the conversions of PNPB were the same (60%) in acetonitrile and supercritical carbon dioxide; the conversion of PNPL was higher in supercritical carbon dioxide (55%) than in acetonitrile (48%). The conversion of PNPP was, however, lower in supercritical carbon dioxide (38%) than in acetonitrile (48%). It should, however, be noted that the conversions in $ScCO_2$ and acetonitrile are significantly higher than observed for PNPL to PNP in aqueous media (less than 1%) [15]. There are several studies that show that enzyme catalyzed reactions in supercritical carbon dioxide yields higher yields than that obtained in conventional organic solvents or solvent-free systems. The faster synthesis of ethyl oleate in supercritical carbon dioxide as compared to that in organic solvents has been reported [64]. Similarly, higher conversions has been reported for the synthesis of oleyl oleate [65, 66], isoamyl acetate [19], isoamyl butyrate [19], ethyl palmitate [17], transesterification of benzyl alcohol, and butyl acetate [67] than in conventional solvents like acetonitrile, hexane, or toluene or under solvent-free conditions. However, there have been a few studies that indicate that some enzyme-catalyzed reactions show better yields in organic solvents compared to that obtained in supercritical fluid systems [68–71]. Alternatively, few studies show that the enzyme activities are comparable/similar in supercritical carbon dioxide and in organic solvents. For example, reaction rates for the esterification of ibuprofen with *n*-propanol [72] and lipase-catalyzed hydrolysis of *p*-nitrophenol butyrate [73] were similar in supercritical carbon dioxide and in *n*-hexane. In this, all the three cases have been observed, i.e., when the conversions are similar, higher, and lower in supercritical carbon dioxide than that obtained in acetonitrile.

## Conclusions

The optimum temperature, reaction kinetics, enzyme loading, and effect of water addition on the hydrolysis of various PNPE in $ScCO_2$ and in acetonitrile were determined using

**Table 1** Kinetic parameters of the Ping Pong bi bi mechanism for the hydrolysis of various *p*-nitrophenyl esters.

| | $V_{max}$ (mol $l^{-1}$ $h^{-1}$) | $K_w$ (mol $l^{-1}$) | $K_e$ (mol $l^{-1}$) | $K_{iw}$ (mol $l^{-1}$) | $K_{ie}$ (mol $l^{-1}$) |
|---|---|---|---|---|---|
| PNPB | 0.005222 | 1.2049 | 0.001671 | 0.42214 | >10 |
| PNPC | 0.003982 | 1.4103 | 0.001924 | 0.43846 | >10 |
| PNPL | 0.001653 | 1.1601 | 0.001486 | 0.4036 | >10 |
| PNPM | 0.001132 | 1.1278 | 0.001476 | 0.41077 | >10 |
| PNPP | 0.00067 | 1.1724 | 0.001513 | 0.39594 | >10 |

Novozym 435. The highest conversion was obtained for PNPB, and the lowest conversion was obtained for PNPP in both acetonitrile and $ScCO_2$ at optimum temperature and enzyme loading. This indicates that the conversion of nitrophenyl esters decreases with increasing chain length. The effect of water addition was significant at lower concentration, but at high water concentration, the equilibrium value was unaffected. An enzyme loading of 3 mg was found to be adequate, and further increase in enzyme loading did not influence conversion significantly. The equilibrium conversion for PNPB, PNPC, PNPL, PNPM, and PNPP were 60, 55, 47, 42, and 38% in $ScCO_2$ and were 60, 48, 58, 53, and 48% in acetonitrile, respectively. The kinetics of the reactions was determined, and the initial velocity for the enzymatic hydrolysis in $ScCO_2$ was modeled successfully using the Ping Pong bi bi mechanism.

**Acknowledgements** The authors thank the Department of Biotechnology, India for financial support.

## References

1. Nini, L., Sarda, L., Comeau, L. C., Boitard, E., Dubes, J. P., & Chahinian, H. (2001). *Biochimica et Biophysica Acta*, *1534*, 34–44.
2. Vaysse, L., Aboubakry, L., Moulin, G., & Dubreucq, E. (2002). *Enzyme and Microbial Technology*, *31*, 648–655.
3. Pencreach, G., & Baratti, J. C. (1996). *Enzyme and Microbial Technology*, *18*, 417–422.
4. Yadav, G. D., & Manjula Devi, K. (2004). *Biochemical Engineering Journal*, *17*, 57–63.
5. Burdette, R. A., & Quinn, D. M. (1986). *Journal of Biological Chemistry*, *261*, 12016–12021.
6. Charton, E., & Macrae, A. R. (1993). *Enzyme and Microbial Technology*, *15*, 489–493.
7. Hui, Y., Wang, S., & Jiang, X. (1982). *Journal of the American Chemical Society*, *104*, 347–349.
8. Gupta, M. N. (1992). *European Journal of Biochemistry*, *203*, 25–32.
9. Harikrishna, S., Manohar, B., Divakar, S., Prapulla, S. G., & Karanth, N. G. (2000). *Enzyme and Microbial Technology*, *26*, 131–136.
10. Chulalaksananukul, W., Condoret, J. S., & Combes, D. (1992). *Enzyme and Microbial Technology*, *14*, 293–298.
11. Klibanov, A. M. (1989). *Trends in Biochemical Sciences*, *14*, 141–144.
12. Turner, C., Persson, M., Mathiasson, L., Adlercreutz, P., & King, J. W. (2001). *Enzyme and Microbial Technology*, *29*, 111–121.
13. Sovova, H., & Ucka, M. Z. (2003). *Chemical Engineering Science*, *58*, 2339–2350.
14. Nagesha, G. K., Manohar, B., & Udaya Sankar, K. (2004). *Journal of Supercritical Fluids*, *32*, 137–145.
15. Srivastava, S., & Madras, G. (2001). *Journal of Chemical Technology and Biotechnology*, *76*, 890–892.
16. Matsuda, T., Harada, T., Nakamura, K., & Ikariya, T. (2005). *Tetrahedron: Asymmetry*, *16*, 909–915.
17. Kumar, R., Modak, J., & Madras, G. (2005). *Industrial & Engineering Chemistry Research*, *43*, 1568–1573.
18. Nakamura, K., Chi, Y. M., Yamada, Y., & Yano, T. (1985). *Chemical Engineering Communications*, *45*, 207–212.
19. Srivastava, S., Modak, J., & Madras, G. (2002). *Industrial & Engineering Chemistry Research*, *41*, 1940–1945.
20. Karel, B. E., & Klibanov, A. M. (1989). *Biotechnology and Bioengineering*, *34*, 1178–1185.
21. Parvaresh, F., Robert, H., Thomas, D., & Legoy, M. D. (1992). *Biotechnology and Bioengineering*, *39*, 467–473.
22. Morild, E. (1981). *Advances in Protein Chemistry*, *34*, 93–166.
23. Lawin, L. R., Fife, W. K., & Tian, C. X. (2000). *Langmuir*, *16*, 3583–3587.
24. Kodaka, M. (1983). *Bulletin of the Chemical Society of Japan*, *56*, 191–2192.
25. Ishiwatari, T., & Fendler, H. (1994). *Journal of the American Chemical Society*, *106*, 1908–1912.
26. Menger, F. M., & Portnoy, C. E. (1967). *Journal of the American Chemical Society*, *901*, 1875–1878.
27. Tee, O. S., & Enos, J. A. (1988). *Canadian Journal of Chemistry*, *66*, 3027–3030.
28. Yang, J., Koga, Y., Hideo, N., & Tsuneo, Y. (2002). *Protein Engineering*, *15*, 147–152.
29. Hayashi, T., & Ikada, Y. (1990). *Biotechnology and Bioengineering*, *36*, 593–600.
30. Macnaughton, S. J., & Foster, N. R. (1994). *Industrial & Engineering Chemistry Research*, *33*, 2757–2763.

31. Chapoy, A., Mohammadi, A. H., Chareton, A., Tohidi, B., & Richon, D. (2004). *Industrial & Engineering Chemistry Research*, *43*, 1794–1802.
32. Perrut, M. (1994). *Chemical and Biochemical Engineering Quarterly*, *8*, 25–30.
33. Condoret, J.-S., Vankan, S., Joullia, X., & Marty, A. (1997). *Chemical Engineering Science*, *52*, 213–220.
34. Srivastava, S., Modak, J., & Madras, G. (2003). *Journal of Supercritical Fluids*, *27*, 55–64.
35. Almeida, M. C., Ruivo, R., Maia, C., Freire, L., Sampaio, T., & Barreiros, S. (2000). *Enzyme and Microbial Technology*, *22*, 494–499.
36. Martins, J. F., Carvalho, I. B., Sampaio, T. C., & Barreiros, S. (1994). *Enzyme and Microbial Technology*, *16*, 785–789.
37. Goddard, R., Bosley, J., & Al-Duri, B. (2000). *Journal of Supercritical Fluids*, *18*, 121–130.
38. Dumont, T., Barth, D., Corbier, C., & Branlant, G. (1992). *Biotechnology and Bioengineering*, *40*, 329–334.
39. Chrastill, J. (1982). *Journal of Physical Chemistry*, *86*, 3016–3021.
40. Nag, A. (1988). *Journal of Surfaces Science and Technology*, *4*, 91–96.
41. Shishikura, A., Fujimoto, K., Suzuki, T., & Arai, K. (1994). *Journal of the American Oil Chemists' Society*, *71*, 961–967.
42. Romero, M. D., Calvo, L., Alba, C., Habulin, M., Primožič, M., & Knez, Ž. (2005). *The Journal of Supercritical Fluids*, *33*, 77–84.
43. Olsen, T., Kerton, F., Marriott, R., & Grogan, G. (2006). *Enzyme and Microbial Technology*, *39*, 621.
44. Gubicza, L., Kabiri-Badr, A., Deoves, E., & Belafi-Bako, K. (2000). *Journal of Biotechnology*, *84*, 193.
45. Güvenç, A., Kapucu, N., & Mehmetoğlu, Ü. (2002). *Process Biochemistry*, *10*, 1–6.
46. Yadav, G. D., & Lathi, P. S. (2005). *Journal of molecular catalysis. B, Enzymatic*, *32*, 107.
47. Chulalaksananukul, W., Condoret, J. S., Delorme, P., & Willemot, R. M. (1990). *FEBS Letters*, *276*, 181–184.
48. Chulalaksananukul, S., Longo, M. A., Chulalaksananukul, W., Condoret, J.-S., & Combes, D. (1999). *AFINIDAD LVI*, *480*, 121–125.
49. Valivety, R. H., Halling, P. J., & Macrae, A. R. (1993). *Biotechnology Letters*, *15*, 1133–1138.
50. Lai, D. T., Hattori, N., & O'Connor, C. J. (1999). *Journal of the American Oil Chemists' Society*, *76*, 845–851.
51. Rizzi, M., Stylos, P., Riek, A., & Reuss, M. (1992). *Enzyme and Microbial Technology*, *14*, 709–714.
52. Straathof, A. J. J., Rakels, J. L. L., Heijnen, J. J. (1992). In J. Tramper, M. H. Vermuë, H. H. Beeftink, U. von Stockar (Eds.), (pp. 137–144). Amsterdam: Elsevier.
53. Valivety, R. H., Halling, P. J., Peilow, A. D., & Macrae, A. R. (1992). *Biochimica et Biophysica Acta*, *1122*, 143–146.
54. Martinelle, M., & Hult, K. (1995). *Biochimica et Biophysica Acta*, *125*, 191–197.
55. Chulalaksananukul, W., Condoret, J. S., & Combes, D. (1993). *Enzyme and Microbial Technology*, *15*, 691–698.
56. Goddard, R., Bosley, J., & Al-Duri, B. (2000). *Journal of Chemical Technology and Biotechnology*, *75*, 715–721.
57. Goddard, R., Bosley, J., & Al-Duri, B. (2000). *Journal of Supercritical Fluids*, *18*, 121–130.
58. Paiva, A. V., Rossum, D. V., & Malcasa, F. X. (2002). *Biocatalysis and Biotransformation*, *20*, 43.
59. Goddard, R., Bosley, J., & Al-Duri, B. (2000). *Journal of Supercritical Fluids*, *18*, 121.
60. Hernandez, F. J., De los Rios, A. P., Gomez, D., Rubio, M., & Villora, G. (2006). *Applied Catalysis B, Environmental*, *67*, 121.
61. Marty, A., Chulalaksananukul, W., Willemot, R. M., & Condore, J. S. (1992). *Biotechnology and Bioengineering*, *39*, 273–280.
62. Rizzi, M., Stylos, P., Riek, A., & Reuss, M. (1992). *Enzyme and Microbial Technology*, *14*, 709–714.
63. Varma, M. N., & Madras, G. (2007). *Industrial & Engineering Chemistry Research*, *46*, 1–6.
64. Yu, Z. R., Rizvi, S. S. H., & Zollweg, J. A. (1992). *Biotechnology Progress*, *8*, 508.
65. Knez, Z., & Habulin, M. (1992). *Progress in Biotechnology*, *8*, 401.
66. Knez, Z., & Habulin, M. (1994). *Biocatalysis*, *9*, 115.
67. Tewari, Y. B., Hara, T., Phinney, K. W., & Mayhew, M. P. (2004). *Journal of Molecular Catalysis. B, Enzymatic*, *30*, 131.
68. Kamat, S., Critchley, G., Beckman, E. J., & Russell, A. J. (1995). *Biotechnology and Bioengineering*, *46*, 610.
69. Vermue, M. H., Tramper, J., Dejong, J. P. J., & Oostrom, W. H. M. (1992). *Enzyme and Microbial Technology*, *14*, 649.
70. Vija, H., Telling, A., & Tougu, V. (1997). *Bioorganic & Medicinal Chemistry Letters*, *7*, 259.
71. Paizs, C., Tosa, M., Bodai, V., Szakacs, G., Kmecz, I., Simandi, B., Majdik, C., Novak, L., Irimie, F. D., & Poppe, L. (2003). *Tetrahedron: Asymmetry*, *14*, 1943.
72. Holmes, J. D., Steytler, D. C., Rees, G. D., & Robinson, B. H. (1998). *Langmuir*, *14*, 6371.
73. Rantakyla, M., & Aaltonen, O. (1994). *Biotechnology Letters*, *16*, 825.