PRESSURE STABILITY OF LIPASES AND THEIR USE IN DIFFERENT SYSTEMS#

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#This paper is dedicated to Professor Dr. Roman Modic

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Abstract

For the investigation of the solvent impact on the enzymes, lipases from the different sources (*Pseudomonas fluorescences, Rhizpous javanicus, Rhizopus niveus, Candida rugosa* and *Porcine pancreas*) were used. Stability and activity of these lipases in aqueous medium in supercritical CO_2 and liquid propane at 100 bar and 40 °C were studied.
On the basis of previous results lipases were used for their application in two different systems. The application of the polysulphone membrane in the continuous stirred tank membrane reactor was studied on the model system of the hydrolysis of oleyl oleate in propane at high pressure. As a catalyst the *Candida rugosa* lipase was used. The next utilization of lipases was the use of on silica aerogel self-immobilized lipase from *Porcine pancreas* as catalyst for esterification reaction in near-critical propane at 40 °C and 100 bar.

Introduction

Many enzymes are stable and catalyze reactions in supercritical fluids, just as they do in other non - or microaqueous environments.[1] Two types of changes in protein structure may occur under extreme conditions. Spatial structure of many proteins is significantly altered and they are denaturated with a loss in activity. On the other hand, at less damaging conditions protein structure is retained on the whole and only local changes occur. Sometimes this local changes lead to another active state of a protein which may posses an altered activity, specificity, and stability.[2]

Enzyme stability and activity depend on the enzyme species, the supercritical fluid, the water content of the enzyme/support/reaction mixture, and on the pressure and temperature of the reaction system.[1]

For the investigation of the solvent impact on enzymes, lipases from different sources were chosen: *Pseudomonas fluorescences, Rhizpous javanicus, Rhizopus niveus, Candida rugosa* and *Porchine pancreas.* The model reactions were esterifications of n-butyric acid with ethanol and iso-amyl alcohol in carbon dioxide and propane. Butyric

acid esters are commonly used as major aroma components of different fruits (pineapple, banana, strawberries, apples, etc).[3]

On the basis of good lipase stability in near critical propane our research work was foccused on combining supercritical fluids and ultrafiltration membranes in developing the membrane bioreactor using polysulphone membrane, operating at high pressure up to 350 bar in propane. A membrane bioreactor is simultaneously accomplishing three unit operations in a single piece of process equipment. The coupling of a biochemical conversion with the separation process is involved in product purification, thus leading to several advantages.

The immobilization of the enzyme on a solid support is one of the most widely employed methods for using them in continuous operated packed bed and stirred tank reactor, especially for large scale operation; it is usually very easy to separate the enzyme from the product solution.[4,5] Since enzymes are normally not soluble in organic or supercritical media there is no need for covalent linkages between the support and the enzyme. It is advantageous to use a porous support so that the enzyme is spread on a large surface area.[6,7] In the present work the application of sol-gel encapsulation technique for the immobilization of lipase from *Porchine pancreas* into silica aerogel matrix and the activity of immobilized lipases in esterification reactions in supercritical fluids have been studied.

Materials and methods

Enzyme preparations

Lipases from: *Pseudomonas fluorescences*, *Rhizpous javanicus*, *Candida rugosa*, *Rhizopus niveus and Porcine pancreas*, were products of Biocatalysis, England.

Lipase activity

The activities of the used lipases were determined as initial rates in esterification reactions of butyric acid with ethanol and iso-amyl alcohol.[8]

Gases

Carbon dioxide 4.5 and propane 3.5 were supplied by Messer MG Ruše, Slovenia.

Synthesis in a Batch Stirred Tank Reactor (BSTR) at atmospheric pressure

Syntheses were performed in a round bottom flask with magnetic stirrer in a water bath at constant temperatures. The volume of the reactor was 50 ml. Initially, the reaction mixture that consisted of butyric acid and alcohol (1:2.5 in mol fractions) was placed into the reactor. Then 0.25 g of enzyme and 2.5 g of water was added. During the reaction the samples were taken from the reactor. The amount of free butyric acid was determined volumetric.[9] Initial reaction rates were determined by monitoring the substrates concentration versus time.

Synthesis in a High Pressure Batch Stirred Tank Reactor (HP BSTR)

The volume of the reactor, designed for operation up to 500 bar and 200 ºC, was 45 ml.[10,11]

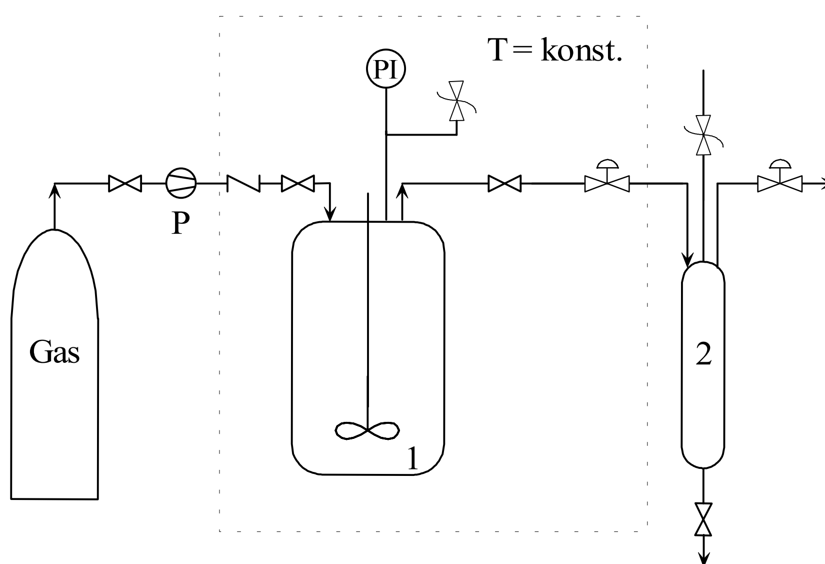

Figure 1. The design of the apparatus for high pressure esterification; 1 – reactor, 2 - separator.

The whole system was termostated at a desired temperature. Initially, the reaction mixture that contained butyric acid and alcohol (1:2.5 in mol fractions) was placed into the reactor. Then 0.25 g of enzyme preparation was added. Finally, CO_2 or propane was pumped into the reactor with a high-pressure pump, up to the desired pressure. During the reaction, samples were taken from the reactor in a trap. The amount of free butyric

acid was determined volumetric.[9] Initial reaction rates were determined as described for batch stirred reactor at atmospheric pressure.

High Pressure Continuous Stirred Tank Membrane Reactor

Polysulfone membranes were prepared (Lab. of organic chemistry of UNI Maribor) by mixing a certain amount of polysulfone in a solvent (dimethylacetamid)[12]. From this solution a solid film of a desirable thickness was formed. The stability of the membranes was evaluated by exposing them to SC-CO_2 and liquid propane at 35 °C and 300 bar. The properties of the membranes, exposed to high pressure and the control membrane (not high pressure treated), were determined with measurements of hydrodynamic resistance and MWCO value by standard methods and then compared. The results showed that there was no difference between the membranes exposed to high pressure and the control membrane. The micro-structure of the membranes were examined with electronic microscope. The top and the bottom surface and crosscut of the membranes that were used in SC-CO_2, propane and those not treated at high pressure were compared.[13] There were no changes in crosscuts, which means that the structure of the membrane remained the same and that also proved their unchanged physical properties. The top and the bottom surfaces of the treated membranes looked different (more compact) with respect to untreated. The reason is probably because the membranes dry out very fast. Water was used as a non-solvent in polysulfone membrane production technology and membranes were usually stored in water. The membranes were also tested on pressure changes stability. Fast and slow pressure changes were performed in a continuous stirred tank membrane reactor and it was found out that the physical properties of the membranes did not change.[14]

The apparatus is presented on Figure 2. The membrane (35 mm diameter) was placed between two sintered plates and fitted in the reactor with 35 mm diameter. A certain amount of the catalyst (hydrated enzyme preparation: 1g water/1g enzyme preparation) was put in the reactor (V=45mL). The autoclave was electrically heated with a heating jacket to ± 0.5 °C. The temperature was measured with an accuracy of ± 0.5 °C with a thermocouple. The substrates and the gas were pumped into the membrane

reactor with the high pressure pump. The pressure was controlled with a manometer Digibar PE 200 Hottinger-Baldwin to ± 0.1%. The products and unreacted substrates were collected in the separator. The catalyst remained in the reactor. Gas left the system after the flow rate measurements.

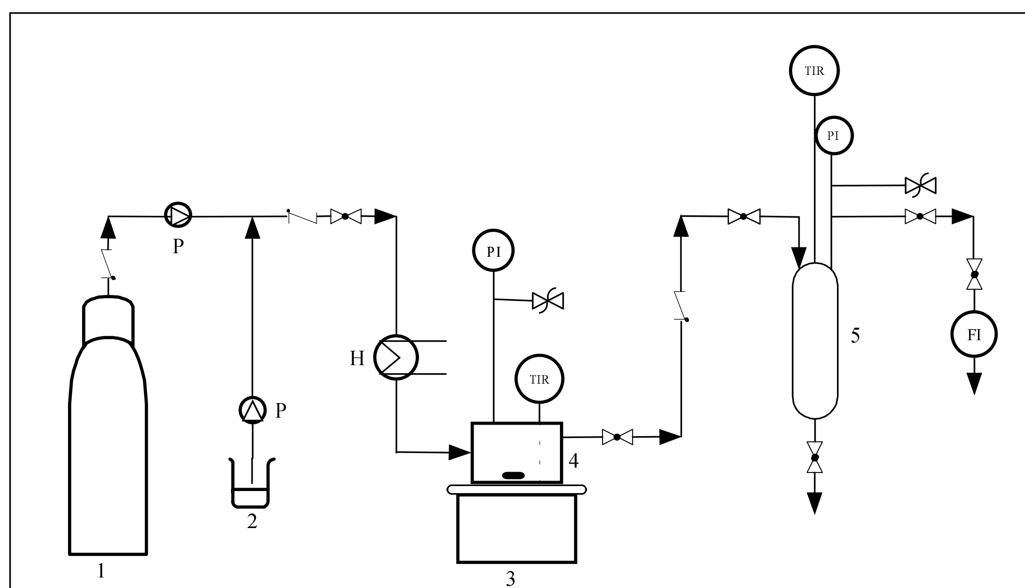

Figure 2. High pressure continuous stirred tank membrane reactor; 1-gas; 2-substrates; 3-magnetic stirrer; 4-membrane reactor; 5-separator; P-high pressure pump; H-heater; PI-pressure indicator; TIR-temperature regulator and indicator.

Results and discussion

Stability of lipases in supercritical carbon dioxide and near critical propane

Before performing reactions in different non-conventional solvents lipases from *Pseudomonas fluorescences, Rhizpous javanicus, Rhizopus niveus* and *Porcine pancreas* were incubated in carbon dioxide and propane at 300 bar and 40 ºC for 24 hours. The same lipase preparations were recovered from the reactor after slow depressurisation and used as catalysts for esterification reactions in aqueous system at 30 ºC and atmospheric pressure. The stability of lipases, previously exposed to dense gases, was determined as a difference in conversion after 3 h between reactions catalyzed by fresh lipases and those catalyzed by lipases, treated with carbon dioxide and propane.

Figure 3 shows the stability data for lipases from *Pseudomonas fluorescences, Rhizopus niveus* and *Rhizopus javanicus,* used as catalysts of esterification between butyric acid and ethanol and for *Porcine pancreas* lipase, for esterification between butyric acid and isoamyl alcohol. The results show that there was no activity change for any of the lipases, when exposed to SC CO_2 and near critical propane. Because of their good stabilities in SC CO_2 and near-critical propane, lipases were used as catalysts in these two media.

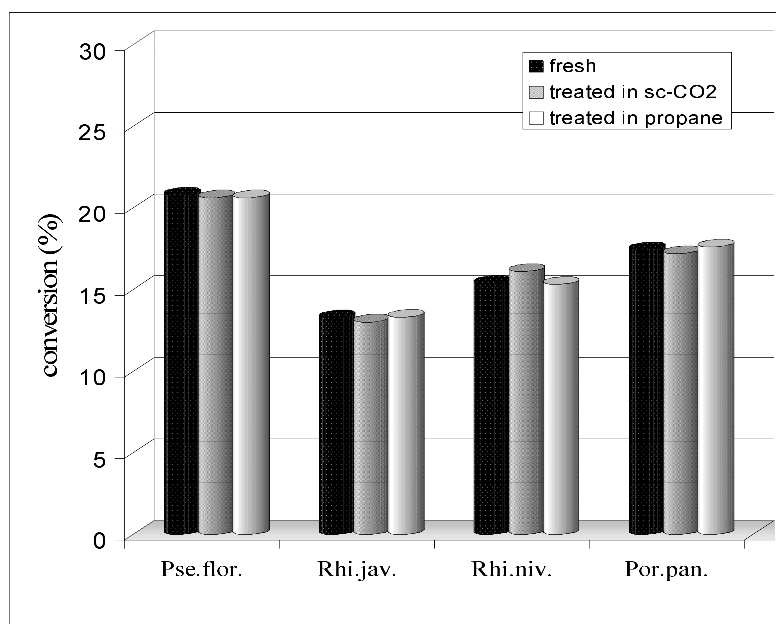

Figure 3. Conversions for esterification reactions of n-butyric acid in aqueous medium at 30ºC and atmospheric pressure, catalyzed by *Pseudomonas fluorescences, Rhizpous javanicus, Rhizopus niveus, and Porcine pancreas* lipases that were previously exposed to SC CO_2 and near critical propane at 300 bar and 40 ºC, and fresh (untreated) lipases.

Thermal stability of Porcine pancreas lipase in water at atmospheric pressure and near-critical propane

The thermal stability of enzymes seems to be generally greater in microaqueous media, including sub- and supercritical fluids (such as propane), than in buffered aqueous solution. To study temperature influence on enzyme stability, esterification between butyric acid and iso-amyl alcohol, catalyzed by *Porcine pancreas* lipase in aqueous medium at atmospheric pressure and in propane at 100 bar in the temperature range from 25 ºC to 60 ºC, was done. As can be seen from Figure 4 the temperature

optimum in aqueous medium - temperature at which the highest initial reaction rate was achieved - moved to a higher value in propane (from 313K in water to 323K in propane). This indicates that the *Porcine pancreas* lipase is more thermostable in propane than in water. This probably is a consequence of the effect of protein structural and conformational rigidity in propane.[15] Protein molecules are generally very labile in water; in organic or, as here, propane media this lability is lost. The outside layer of amino acids is damaged and forms a sort of a protection layer for protein on the inner side of the molecule. Therefore temperature does not denaturize protein as fast as it happens in aqueous medium.

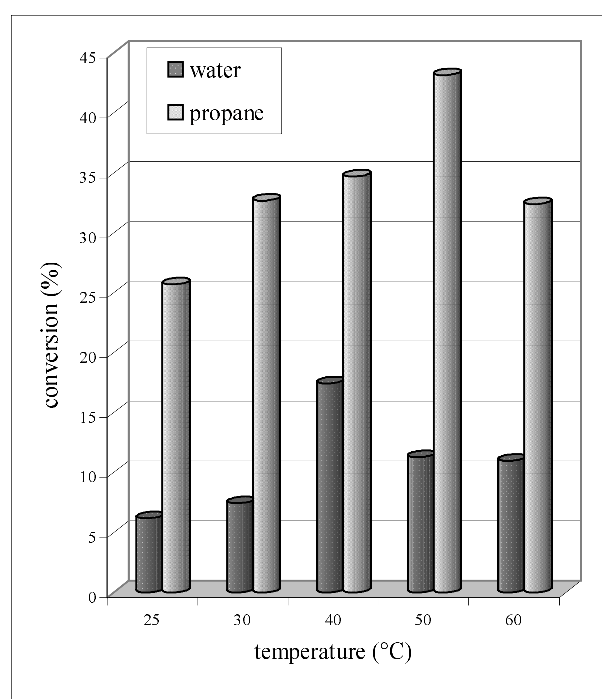

Figure 4. Thermal stability of *Porcine pancreas* lipase; esterification reaction between n-butyric acid and iso-amyl alcohol in aqueous medium and atmospheric pressure, and in near-critical propane at 100 bar.

Thermal activation and deactivation (energy of activation and deactivation enthalpy respectively) were determined from the Arrhenius diagram. Results are presented in Table 1. Deactivation constant for enzyme is expressed as the ratio between the amount of enzyme in its inactive and active form at the temperature at which the

initial reaction rate was the greatest. The calculated Kd showed that there was more than twice as much of inactive enzyme as active in examined enzyme preparation in water while this ratio is 1 in propane. As can be seen the higher Ea and ΔHd in water indicates that enzyme activity was more influenced by temperature in water than in propane. The deactivation entrophy and free Gibb's energy of deactivation were also determined by using the expression:[16]

$$Kd(T_{max}) = (E+RT_{max}) / (\Delta Hd-E/RT_{max}) \quad (1)$$

$$Kd = \exp(-\Delta Gd/RT) = \exp(\Delta Hd/RT)*\exp(\Delta Sd/R) \quad (2)$$

As the temperature at fixed pressure was increased the density of the compressible solvent propane decreased. These results suggest that the activation of lipases by temperature at constant pressure is solely a temperature effect in propane whereas this temperature effect is combined with one mediated by changes in solvatation due to the changes in density.

Table 1. Energy of activation (Ea), deactivation enthalpy (ΔHd) and deactivation constant (Kd) for esterification between butyric acid and iso-pentanol, catalyzed by *Porchine pancreas* lipase in aqueous medium at atmospheric pressure and in near critical propane at 100 bar.

	Ea(J/mol)	**ΔHd(J/mol)**	**Kd(T_{max})**
aqueous medium	94945	140598	2.26
propane	26072	57847	0.98

Hydrolysis of oleyl oleate in a high pressure continuous stirred tank membrane bioreactor

On the basis of previous results our research work was oriented in combining supercritical fluids and ultrafiltration membranes in developing the membrane reactor using polysulfone membrane, operating at high pressure up to 350 bar in propane.

The application of the polysulfone membrane in the reaction system was studied on the model system of the hydrolysis of oleyl oleate ester (ester of oleic acid and oleyl alcohol) in near critical propane. The hydrolysis was performed in the continuous stirred tank

membrane reactor at 300 bar and 35 °C. As a catalyst the *Candida rugosa* lipase was used. The aim of our experiments was to separate the enzyme (water soluble protein, molecular weight around 50000 g/mol) from the reaction mixture (ester, water buffer and propane) for the purpose of the enzyme reuse in the reactor. The time course of the reaction is presented on Figure 5.

It was found out that the conversion reaches its equilibrium after 100 minutes and remains the same for another 300 minutes. That means that the catalyst (enzyme) retained in the reactor and the separation was succesfully performed which was prooved by the unchanged level of the conversion.

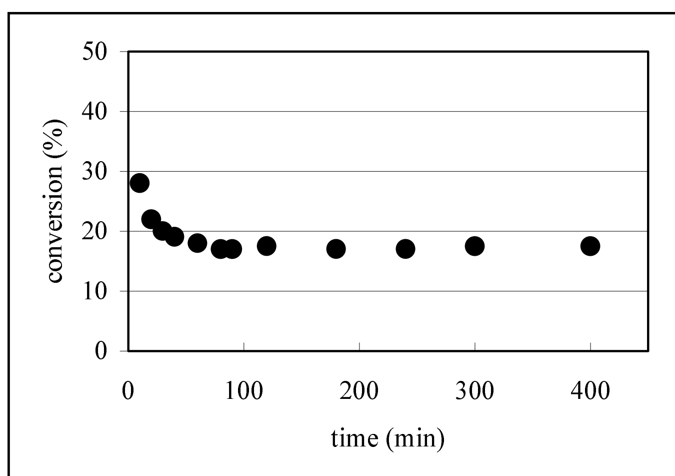

Figure 5: Time course of ester hydrolysis performed in continuous stirred tank membrane reactor operating at high pressure of propane (300 bar, 35 °C) using lipase from *Candida rugosa* as catalyst. Flow rate of propane was 0,5 L/min and flow rate of reactants was 1,5 mL/min.

Silica aerogel as a support for enzyme catalyzed reaction

The next utilization of enzymes was the use of self-immobilized lipase from *Porcine pancreas* on silica aerogel.[17] Such immobilized lipase was used for catalyzing the esterification reaction between butyric acid and iso-amyl alcohol in supercritical CO_2 and near critical propane at 40 °C and 100 bar.

The results presented as the conversion depending on the type of medium and enzyme preparations are on Figure 6 and can be explained as follows:

- Relative activity in SC-CO_2 is 25. The immobilized lipase on silica aerogel is more active than free lipase from the same source because immobilized lipase is entrapped and therefore protected in sol-gel matrix. Supercritical CO_2 as a reaction medium deactivates free lipase.
- Relative activity in propane is 2.5. The immobilized lipase is 2.5 times more active in propane than free enzyme. The free lipase is not soluble in propane. It forms aggregates, which have less accessible active sites. With the immobilization the enzyme is spread out in the matrix pores and more active sides are accessible. The result is a higher conversion.

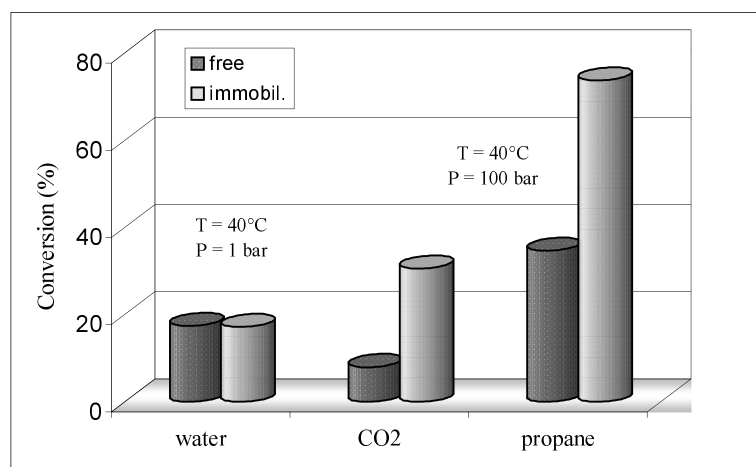

Figure 6. Influence of the immobilization and reaction medium on the conversion; esterification between butyric acid and iso-amyl alcohol, catalyzed by the *Porcine pancreas* lipase.

When immobilization techniques are studied, the data about the stability of immobilized enzyme preparation are very important. Figure 7 shows the changes in conversions of the esterification reactions between butyric acid and iso-amyl alcohol with the number of reaction cycles. One cycle means one reaction performed in the batch stirred tank reactor, which lasted for 6 h. After each reaction enzyme preparation was washed three times with hexane and water.

For our model reaction, esterification between butyric acid and isoamyl alcohol, propane seems to be more appropriate solvent than SC CO_2. In the combination with immobilized lipase from *Porcine pancreas* on silica aerogel the conversion is 75%. After the fourth cycle the conversion is still above 50%, while the conversion in SC CO2 is much lower. After fifth cycle the conversion is comparable with the one in SC CO_2. It remains stable and is still higher than in aqueous medium.

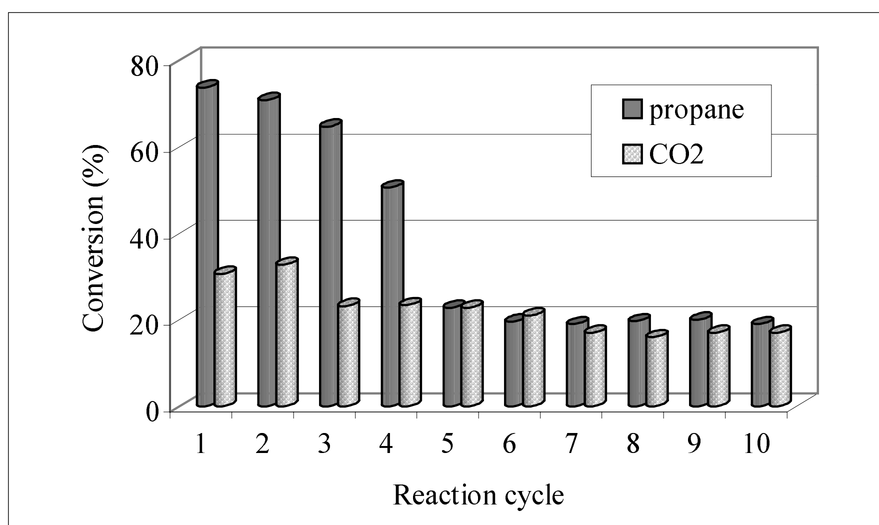

Figure 7. Stability of the immobilized *Porcine pancreas* lipase in SC CO_2 and near-critical propane.

Conclusions

Lipases from *Pseudomonas fluorescences, Rhizpous javanicus, Rhizopus niveus*, and *Porcine pancreas* are stable in supercritical carbon dioxide and near-critical propane (300 bar and 40 ºC). Their activity remains the same even after 24 hours of incubation at mentioned conditions.

In near-critical propane thermal stability of the *Porcine pancreas* lipase is highly improved.

Polysulphone membranes were succesfully used as a separation unit in the continuous stirred tank membrane bioreactor operating at 300 bar and 30 °C in propane atmosphere.

The immobilization technique of lipase from from *Porcine pancreas* into silica aerogel matrix has proved to be very applicable when supercritical or near-critical fluids

are used as reaction media. With the immobilization the deactivation of free enzyme in SC CO_2 and agglomeration in near-critical propane is overcome. The reason for very high relative enzyme activities in the case of dense gases as reaction media is high dispersion of the lipases in the silica aerogel matrix.

References and Notes

1. Aaltonen, O. In: *Chemical synthesis using supercritical fluids*; Eds.: Jessop, P.G., Leitner, W., 1999, p. 414.
2. Mozhaev, V.V.; Kudryashova, E.V.; Bec, N. *High Pressure Bioscience and Biotechnology*; Eds.: Hayashi, R.; Balny, C., Elsevier Science, 1996, p 221.
3. Burger, A.M. In: *Die natürlichen und künstlichen Aromen*, Dr. Alfred Huting Verlag, Heidelberg, 1968, p 130.
4. Reetz, M.T. *Advanced materials* **1997**, *9*(12), 943.
5. Reetz, M.T.; Zon, A.; Simpelkamp, J.; Rufinska, A.; Tesche, B. *J. of Sol-Gel Sci. and Technol.* **1996**, *7*, 35.
6. Ballestros, A.; Bornscheuer, U.; Capewell, A.; Combes, D.; Condoret, J.S.; Koenig, K.; Kolisis, F.N., Marty, A.; Menge, U.; Schepper, T.; Stamatis, H.; Xenakis, A. *Biocatalysis and Biotransformations* **1995**, *13*, 1.
7. Cabral, J.M.S.; Best, D.; Barros, L.; Tramper, *J. Applied Biocatalysis*; Harwood Academic Publisher: Switzerland, 1994.
8. Habulin, M.; Krmelj, V.; Knez, Ž. *Fifth Conference on Supercritical Fluids and Their Applications*, Garda, Verona, Padova, 1999, pp. 331-337.
9. Leitgeb, M.; Knez, Ž. *J. Am. Oil Chem. Soc.* **1990**, *67*, 775-778.
10. Knez, Ž.; Habulin, M.; Krmelj, V. *J Supercrit. Fluids.* **1998**, *14*, 17-19.
11. Habulin, M.; Krmelj, V.; Knez Ž. *J. Agric. Food. Chem.* **1996**, *44*(1), 338-342.
12. Kaiser, V.; Stropnik, Č. *Kovine zlit. tehnol.* **1999**, *33*(3-4), 215-218.
13. Cheryan, M. In: *Ultrafiltration handbook*; Publishing Company, Lancaster, 1986, p. 53.
14. Krmelj, V.; Habulin, M.; Vasič-Rački, Đ.; Knez, Ž. In: *Proceedings of the 5th International symposium on supercritical fluids*, Atlanta, Georgia, USA, 2000.
15. Koskinen, A.M.P.; Klibanov, A.M. *Enzymatic reactions in organic media*, Blackie Academic & Professional, London, 1996.
16. Bailey, J.E.; Ollis, D.F. In: *Biochemical engineering fundamentals*; Eds.: Carberry, J.J.; Fair, J.R.; Peters, M.S.; Schowalter, W.R.; Wei, J. Mc Graw-Hill Book Company, New York, 1986, p 86.
17. Novak, Z.; Krmelj, V.; Habulin, M.; Knez, Ž.; Bauman, D. In: *Proceedings of the 5th International symposium on supercritical fluids*, Atlanta, Georgia, USA, 2000.

Povzetek

Za študijo vpliva topil na encime smo izbrali lipaze iz raznih virov (*Pseudomonas fluorescences, Rhizopus javanicus, Rhizopus niveus, Candida rugosa* in *Prochine pancreas*). Ugotavljali smo spremembe v aktivnosti oz. stabilnosti omenjenih lipaz v vodnem mediju pri atmosferskem tlaku, v superkritičnem CO_2 in sub-kritičnem propanu.

Na osnovi dobljenih rezultatov smo lipaze uporabili kot katalizatorje v dveh različnih sistemih. Uporabo polisulfonskih membran v kontinuiranem mešalnem membranskem reaktorju smo študirali s pomočjo modelnega sistema hidrolize oleil oleata v sub-kritičnem propanu. Kot katalizator smo uporabili lipazo iz *candida rugose*. Druga aplikacija pa je bila uporaba *Porchine pancreas* lipaze, ki smo jo predhodno sami imobilizirali na silicijevem aerogelu, za katalizo esterifikacije v sub-kritičnem propanu