Mini Review

Supercritical Fluids as Solvents for Enzymatic Reactions

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Dedicated to the memory of professor Vojko Ozim

Abstract

Enzymes may act in different solvent systems. Water as the solvent *in vivo* may be replaced partially or mostly with other solvents, such as micro-emulsions, organic solvents, reversed micelles, ionic liquids and supercritical fluids (SCFs). Several types of enzymatic reactions were performed in SCFs. Influence of SCFs on enzyme stability and activity is presented on different examples; on different reaction systems (hydrolysis, transesterification …) and on the use of non-immobilized (*Subtilisin carlsberg, Aspergillus niger* …) as well as immobilized enzymes. Several types of high-pressure enzymatic reactors (batch-, stirred-tank-, extractive semibatch-, recirculating batch-, semicontinuous flow-, continuous packed-bed-, and continuous-membrane reactors) have been used for the performance of enzymatic reactions. In the studies on stability of biocatalysts in a high-pressure batch-stirred tank reactor changes in biocatalysts activity due to pressurization/depressurization steps were observed. Interesting alternative to overcome this inconvenience is the use of the high-pressure continuous membrane reactors, where just single compression and expansion step is necessary.

Keywords: Enzymatic reactions, supercritical carbon dioxide, high-pressure, enzyme stability, high-pressure reactors …

1. Introduction

Biocatalysis will be in the future the most efficient way of producing fine chemicals, especially to avoid such a huge amount of wastes, produced today with conventional production methods.

Enzymes may act in different solvent systems. Water as the solvent *in vivo* may be replaced partially or mostly with other solvents, such as microemulsions, organic solvents, reversed micelles, ionic liquids and SCFs. Sometimes the solvents are combined, e.g. ionic liquids may be combined with SCFs to reach the most efficient way for separation, needed at the end of the reaction. The use of enzymes acting as catalysts in nonaqueous media has been widely described in the scientific literature.[1,2] The interest of using biocatalysts in SCFs has been growing rapidly in recent years, mainly in industrial and pilot applications.[3]

Enzymatic reactions in SCFs have been studied in the past two decades very intensively.[4–6] Many of enzymatic reactions have been performed in supercritical carbon dioxide (SC CO_2) with different kinds of enzymes. Still mostly hydrolases have been used as biocatalysts for the reactions, performed in SCFs.

Usually high-pressure batch reactors were used for the screening of the enzymes and for determination of kinetics of enzymatic reactions. Some reactions were also performed in continuous reactors. Among these, continuous packed-bed reactors were used for immobilized enzymes. In the latest time high-pressure membrane reactors with bio-active membranes are used.

2. Enzymatic Reactions in SCFs

Physical properties of SCFs are very sensitive to small changes in temperature and/or pressure in the vicinity of the critical point. This means that, for example, with small changes of temperature and/or pressure near the critical point significant changes in the density of a SCF occur. Therefore the solvent power of a SCF may be tuned this way.

SCFs may be used for extractions, micronization, chromatography and finally for chemical and biochemical reactions.

Table 1: Some examples of enzymatic reactions, performed in SC CO_2 and other organic and inorganic solvents.

Substrates	Solvent	Enzyme	Reaction	Type of Reactor	Authors
1-phenylethanol, vinyl acetate	SC CO_2, SC SF_6	*Pseudomonas cepacia* lipase	transesterification	batch reaction cell	Celia et al. (2005)[7]
Isoamyl alcohol, Acetic acid or Ammonium acetate or Ethyl acetate or Acetic anhydride	SC CO_2, *n*-hexane	*Candida antarctica* lipase, *Rhizomucor miehei* lipase	esterification	stirred-batch reactor, continuous tubular reactor	Romero et al. (2005)[8]
Hydrolysed soy deodorizer distillate, butanol	SC CO_2	*Mucor miehei* lipase	esterification	stirred-batch reactor	Nagesha et al. (2004)[9]
Benzyl alcohol, butyl acetate	SC CO_2, *n*-hexane, toluene, neat media	*Candida antarctica* lipase	transesterification	modified supercritical fluid chromatograph	Tewari et al. (2004)[10]
rac-glycidol, vinyl acetate or vinyl butyrate	SC CO_2, Ionic liquids	*Candida antarctica* lipase, *Mucor miehei* lipase	transesterification	continuous reactor	Lozano et al. (2004)[11]
1-butanol, vinyl butyrate	SC CO_2, *n*-hexane	*Candida antarctica* lipase	transesterification	stirred continuous membrane reactor	Lozano et al. (2004)[12]
Poly(azelaic anhydride) 1,8-octanediol	SC CO_2	*Candida antarctica* lipase	polymerization	batch reactor	Uyama et al. (2003)[13]
Myristic acid, ethanol	SC CO_2	Lipase – Crude HPL	esterification	batch reactor	Sirvastava et al. (2003)[14]
1-(*p*-chlorophenyl)-2,2,2-trifluoroethanol, vinyl acetate	SC CO_2	*Candida antarctica* lipase, *Rhizomucor miehei* lipase, *Pseudomonas cepacia* lipase, *Pseudomonas aeruginosa* lipase	acetylation	batch reactor	Matsuda et al. (2003)[15]
2-benzyl-1,3-propanediacetate, methanol	SC CO_2	*Candida antarctica* lipase	asymmetrization	batch reactor	Mase et al. (2003)[16]
Blackcurrant oil, distilled water	SC CO_2	*Mucor miehei* lipase	hydrolysis	continuous flow reactor	Sovova et al. (2003)[17]
Vinyl octanoate, 3-methyl-2-butanol	SC CO_2	*Candida antarctica* lipase	transesterification	batch reactor	Ottosson et al. (2002)[18]
1-(*p*-chlorophenyl)-2,2,2-trifluoroethanol, vinyl acetate	SC CO_2	*Candida antarctica* lipase	acetylation	batch reactor	Matsuda et al. (2001)[19]
3-hydroksy-5-phenyl-4-pentenoicacideth ylester	Biphasic KKP-buffer/ SC CO_2 system	*Pseudomonas cepacia* lipase, porcine pancreas lipase	hydrolysis	continuous membrane reactor	Hartmann et al. (2001)[20]
Stearic acid, ethanol Ethyl stearate	SC CO_2	*Mucor miehei* lipase	esterification hydrolysis	batch reactor	Nakaya et al. (2001)[21]
Oleic acid, ethanol, water	SC CO_2	*Rhizomucor miehei* lipase	esterification	packed-bed continuous reactor	Al-Duri et al. (2001)[22]
1,2,3-trioleoyl glycerol, ethylbehenate, water	SC CO_2	*Mucor miehei* lipase	hydrolysis, transesterification	batch reactor	Yoon et al. (1996)[23]
Hydroxyoctanoic acid methyl ester, vinyl acetate	SC CO_2	*Pseudomonas cepacia* lipase	acylation	batch reactor	Bornscheuer et al. (1996)[24]
3-hydroxyoctanoic acid methyl ester, vinyl acetate	SC CO_2	*Pseudomonas cepacia* lipase	acylation	batch reactor	Capewell et al. (1996)[25]

Substrates	Solvent	Enzyme	Reaction	Type of Reactor	Authors
n-valeric acid, citronellol, oleic acid, citronellol	SC CO_2	*Candida cylindracea* lipase	esterification	continuous flow type reactor	Ikushima et al. (1996)[26]
Oleic acid, citronellol	SC CO_2	*Candida cylindracea* lipase	esterification	continuous flow type reactor	Ikushima Yutaka (1997)[27]
Isoamyl alcohol + ammonium acetate (acetic acid)	*n*-hexane, SC CO_2	Lipase (Novozym, Lipolase)	acylation	batch reactor	Vija et al. (1997)[28]
2-alkanone (2-butanone, 2-pentanone, 2-hexanone, 2-heptanone, 2-octanone) and 2-propanol	*n*-hexane, toluene, methyl tert-butyl ether, SC CO_2	Ketoreductase	transhydroxylation	batch reactor	Tewari et al. (2005)[29]
2-*exo*-bromo-3-*endo*-hydroxybicyclo [3.2.0]heptan-6-one	SC CO_2	Lipolase	hydrolysis	batch reactor	Parve et al. (1997)[30]
Triolein, 1,3-diolein, *rac*-1,2-diolein, *rac*-1-monoolein, water	SC CO_2	porcine pancreas lipase	hydrolysis	batch reactor	Glowacz et al. (1996)[31]
Retinyl palmitate, α-tocopheryl acetate	di-isopropyl ether, hexane/ ethanol, SC CO_2 (+ ethanol)	Lipases from *Candida antarctica, Rhizomucor miehei, Pseudomonas cepacia*	hydrolysis, alcoholysis	continuous reactor	Turner et al. (2001)[32]
Ethanol, cod liver oil	SC CO_2	Lipase from *Candida antarctica*	ethanolysis	extraction reactor (ethanol introduced into the reactor continuous or batch)	Gunnlaugsdottir et al. (1998)[33]
Ethanol, cod liver oil	SC CO_2	Lipases from *Humicola lanuginose, Candida antarctica* lipase B	alcoholysis	continuous reactor	Gunnlaugsdottir et al. (1998)[34]
Castor oil triglycerides, methyl oleate	SC CO_2	Lipase from *Candida antarctica*	interesterification	batch reactor, enzymatic membrane reactor	Pomier et al. (2005)[35]
Different fatty acids, isoamyl alcohol	SC CO_2	Hog pancreas lipase, Lipolase 100T, Novozym 435	esterification	batch reactor	Kumar et al. (2005)[36]
p-chiral hydroxymethane phosphinates, vinyl acetate	SC CO_2	Lipases LPL, AK, PS-C, Lipozyme and CAL (Novozym)	acetylation	batch reactor	Albrycht et al. (2005)[37]
Sunflower oil, alcohol	SC CO_2	Lipase Novozym 435	transesterification	batch reactor	Madras et al. (2004)[38]
Racemic 1(*p*-chloro-phenyl)-2,2,2-trifluoroethanol, vinyl acetate	SC CO_2, ionic liquid	Lipase Novozym	enantioselective acetylation	batch reactor, flow reactor	Matsuda et al. (2005)[39]
Oleic acid, *n*-butanol	SC CO_2	Lipase from *Rhizomucor miehei*	esterification	batch reactor	Habulin (1993)[40]
Isoamyl alcohol, acetic anhydride	SC CO_2	Lipase Novozym 435	esterification	continuous packed-bed reactor	Romero et al. (2005)[41]
Sunflower oil	SC CO_2	Lipase Lipolase 100T	hydrolysis	batch reactor, membrane reactor	Primožič et al. (2003)[42]

Different compounds may be used as SCFs. Among inorganic compounds SC CO_2 has been mostly used up till now and in these days processes with supercritical water (SCWO) are developed (but not for enzymatic reactions). Among organic compounds propane in the sub-critical state is the most appropriate for the use as a solvent for enzymatic reactions. Application of "green" freons (e.g. R 134a (1,1,1,2-tetrafluoroethane) …) in the supercritical state as a solvent for enzyme-catalyzed reaction has been growing also in the last years.

Several enzymatic reactions, such as oxidation, hydrolysis, transesterification, esterification, enantioselective synthesis were performed in SCFs (Table 1).

Using SC CO_2 as a process medium has several advantages, such as: unique transport properties, adjustable solvent power of the solvent and design of a production process with integrated downstream separation of products and unreacted substrates. Additional advantages of using SC CO_2 as a medium for enzymatic catalyzed reactions have been well documented:[43–45] its critical pressure (7.38 MPa) is "acceptable" and its critical temperature (31.1 °C) is consistent with the use of enzymes and/or labile solutes. Moreover, SC CO_2 performs mainly as a lipophylic solvent; it is cheap, not toxic and non-flammable. In addition, its "naturalness" is greatly appreciated by the food and health-care related industries. Their capacity of encouraging transport phenomena (due to high diffusivities) and facilitate reaction products separation by tuning solvent power make the SCFs, such as SC CO_2, extremely attractive to use as 'green-designer' solvents for environmentally more acceptable chemical processes.[4–6, 46,47] However, it has also disadvantages, as sometimes lower catalytic activities in the solvent which have been attributed to the formation of carbonic acid.

For the use of enzymes as biocatalysts in SCFs their advantage that they are not bound to their natural environment (they exhibit a high substrate tolerance and are not specifically required to work in water) is very important. The advantage that they act under mild conditions (pH about 5–8, typically around 7 and in a temperature range of 20–40 °C) may be sometimes turned into a drawback.

Enzymes as biocatalysts require narrow operation parameters. Elevated temperatures, as well as extreme pH, may lead to deactivation of the protein.

Many enzymatic reactions are prone to substrate or product inhibition. Therefore enzymes work at lower substrate or product concentrations, which limit the efficiency of the process. Whereas substrate inhibition can be circumvented easily by keeping the substrate concentration at low level through continuous addition, product inhibition is a more complicated problem. The gradual removal of product by physical means is usually difficult, but can be done elegant with the use of dense gases as reaction media in a continuous process.

Enzymes are proteins designed to fit a specific substrate(s). They have the active site which is tailor-made for the substrate. In a dense gas the enzyme molecule is becoming more rigid. This rigidity may be an advantage in the case of protein deactivation, namely the enzyme molecule is not prone to denaturation so quickly. At high pressure, spatial structure of many proteins may be significantly altered and they are denaturated with a loss in activity.

In SCFs there are direct effects of pressure on enzyme activity, which may lead to denaturation and indirect effects of pressure on enzymatic activity. In the case of SC CO_2 only small direct effects of pressure with regard to enzyme inactivation are expected. Protein structure should retain on the whole and only local changes may occur. Those local changes may lead to another active state of a protein which may posses an altered activity, specificity, and stability. Pressure is also likely to affect the reaction performance indirectly by changing either the rate constant or the reactants solubility. At higher pressures more solute-solvent interactions take place, resulting in a better solvent capacity.

Enzyme stability and activity in dense gases depend on: the source of the enzyme, the SCF, the water content of the enzyme/support/reaction mixture and the pressure and temperature of the reaction system. Therefore no theoretical prediction whether an enzyme should be active in a SCF or not can be made. Experimental study of the system behaviour is necessary.

Enzymes in SCFs could be used in their native form (powder, liquid ...) or immobilized on a carrier (resin, sol-gel matrix ...). No matter in which shape the enzymes are used, biocatalysis in SC CO_2 is always heterogeneous, because enzymes are not soluble in CO_2. The stability of an enzyme is dependent from the shape of the enzyme. Immobilization methods (physical or chemical) also influence enzyme stability and activity.

3. Stability of Enzymes in SCFs

Lipases, if incubated in different dense gases are usually not sensitive to the influence of the medium which is not valid for other enzymes. However, the choice of a medium is very important if enzymes are used as biocatalysts in these media.

3. 1. Non-immobilized Enzymes

Non-immobilized lipases from different sources were first incubated at defined conditions in SC CO_2 and in sub-critical propane and afterwards used as biocatalysts. The comparison with the reaction, catalysed by non-incubated (fresh) enzyme showed that these lipases are stable in the two examined media.[48]

Lipases from the same sources as in previous case in their non-immobilized form were used as biocatalysts

for esterification between butyric acid and ethanol in CO_2 and propane at high pressure. Initial reaction rates strongly depend upon the choice of the solvent used for the reaction performance. Not only changed enzyme activity caused differences in initial reaction rates. Other effects, such as water partition between the enzyme and the reaction mixture, solvent power of the reaction medium or dielectric constant affected rate of reaction, too. Activity of biocatalysts is strongly dependent on the dielectric constant of the solvent.[49,50] A major change in protein flexibility occurs when solvent dielectric constant increases from 1 to 10. It has been suggested that dielectric constant can be used to predict the specificity of an enzyme-catalyzed reaction. Russell et al.[51] showed that in a *Subtilisin carlsberg*-catalyzed, or *Aspergillus* protease-catalyzed, transesterification reaction between N-acetyl-(L or D)-phenylalanine ethyl ester and methanol pressure-induced changes in the dielectric constant of fluoroform gave rise to predictable changes in the enantioselecivity of both enzymes.

Activity of the in SC CO_2 (at 30 MPa) preincubated non-immobilized proteinase from *Carica papaya* latex changed in the comparison to the activity of the crude enzyme preparation.[52] These changes were connected with water distribution in the system. The crude proteinase contained 1.53% of water, while the proteinase incubated in SC CO_2, contained only 0.99% of water. The wt. % of present water was determined by Karel-Fisher method. Water plays a vital role in the non-covalent interactions that allow the enzyme to retain its native conformation. In the complete absence of water, enzymes cannot maintain an active conformation, thus hindering their ability to function as catalysts.[53] In SC CO_2 at temperatures above 40 °C the proteinase activity decrease appeared, while at atmospheric pressure better temperature stability of the biocatalyst was observed.

The residual activity of proteinase, which was incubated in near-critical propane and dimethyl ether (DME) at 30 MPa, was lower than the activity of the crude enzyme preparation. In DME the original value was reached with the thermal activation at 50 °C and 60 °C. It is evident that in this case physical properties of SCFs have a dramatic effect on enzyme stability.

3. 2. Immobilized Enzymes

Immobilized enzymes are expected to be more stable in sub- and supercritical media and therefore their activity should be almost unchanged.

Immobilized lipase from *Rhizomucor miehei* was first incubated in different gases (SC CO_2, sub-critical butane and mixture of *n*-propane/*n*-butane) at 35 °C and 10 MPa and afterwards used as a biocatalyst. No difference in the residual activity was observed.[54] When the same lipase was used as biocatalyst in the same dense gases as before at 50 °C and pressure from 10 MPa to 30 MPa, the highest reaction rates were achieved in SC CO_2. In *n*-butane and in the mixture of *n*-propane/*n*-butane reaction rates were almost the same. At given temperature and pressure *n*-butane and the mixture of *n*-propane/*n*-butane are at sub-critical conditions, while CO_2 exists at supercritical state. Reaction rates in *n*-butane and in the mixture of *n*-propane/*n*-butane did not change with the pressure rise from 10 MPa till 30 MPa while in the same pressure range initial reaction rates in SC CO_2 increased with higher pressure for about 70%.

4. Effect of the Medium on Particle Size of Biocatalyst

The size of the enzyme preparation particle varies with solvent since different solvents promote clustering of enzyme particles to different degrees.[51] In SCFs, one expects that enzyme powders would undergo morphological changes, which depend on the solvent, temperature and pressure.[53]

Lipase Novozym 435 from *Candida antarctica B* was incubated in SC CO_2 for 24 hours at 60 °C and at 10 MPa. For the comparison with an organic solvent the same lipase was incubated in 2-methyl 2-butanol at the same temperature as before, as well. Particle size distribution of the untreated lipase and lipase which was previously treated in the solvent was determined. Figure 1 shows the change of the mean particle size by treatment with SC CO_2 or 2-methyl 2-butanol.

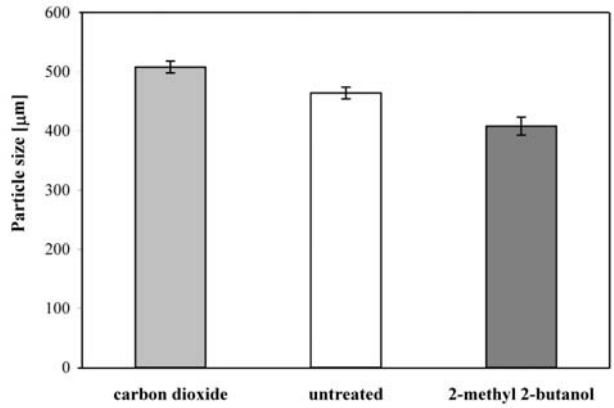

Figure 1: Mean particle size of *Candida antarctica B* lipase exposed to the different solvents.

The mean particle size of lipase treated in 2-methyl 2-butanol was smaller (408 µm) compared to the untreated lipase (464 µm). The reason for the decrease in mean particle size of lipase could be that 2-methyl 2-butanol is deleterious to immobilized *Candida antarctica B* lipase preparation, resulting in partial dissolving of its carrier. On the contrary, the mean particle size of lipase treated in

SC CO_2 was bigger (508 μm). This phenomenon could be explained by swelling of the immobilized lipase.

The analyses of the change of the lipase morphology by treatment with SC CO_2 or 2-methyl 2-butanol were performed by Scanning Electron Microscope (SEM). The images of untreated lipase and lipase treated with SC CO_2 and 2-methyl 2-butanol are shown in Figure 2. SEM images of untreated and treated lipase showed, that the morphology of the immobilized lipase is not affected by 2-methyl 2-butanol or SC CO_2 at selected conditions within 24 hours.

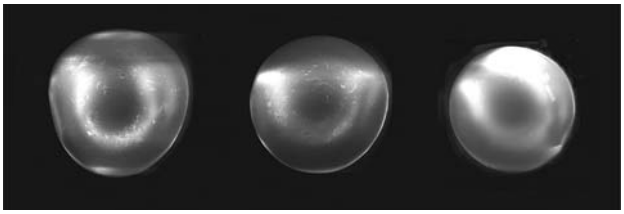

Figure 2: SEM of untreated (left) and treated lipase from *Candida Antarctica B* (middle: treated with SC CO_2, right: treated with 2-methyl 2–butanol).

4. 1. Effect of Water on Enzyme Activity in SCFs

Water is crucial for enzymes and affects enzyme action by influencing enzyme structure via noncovalent binding and disruption of hydrogen bonds, by facilitating reagent diffusion and by influencing the reaction equilibrium. Use of an enzyme in pure SC CO_2 may lead to the removal of water, which is included or bonded to the enzyme. The quantity of the removed water is temperature and pressure depended.

When an enzyme is used in SC CO_2 in a batch system with each expansion a certain amount of water is removed from the enzyme preparation. To avoid enzyme deactivation as a consequence, water could be added to the system at the start of the reaction. The optimal initial water concentration should be determined for each bioreaction system because even small differences of initial water concentration may cause big differences in enzyme action.

Therefore the influence of added water on the activity of proteinase from *Carica papaya* was studied.[52] Optimal water concentration at 30 MPa and 50 °C was found to be between 0.5 g/L and 0.7 g/L, where the residual activity of the proteinase increased for 17.5%. With higher amount of water activity of the proteinase slowly decreased. With increase in added water till 1.0 g/L, the activity of exposed enzyme preparation was still equal to the activity of the original sample, where no water was added. Water concentration above 1.0 g/L caused the drop in residual activity for 80%.

5. Effect of Temperature and Pressure on Enzyme-catalyzed Reactions in SCFs

Pressure is likely to affect the reaction rate by changing either the reactants solubility or the rate constant directly. Indeed, an increase in pressure leads to enhanced fluid density and, therefore, improved solvating power of the fluid. On the one hand, the solubility of substances increases with higher pressures because of a higher fluid density and this is essential to bring the initial products in the reactor and remove the end products from the reactor. Therefore a pressure increase is, in most cases, positive for enzymatic reactions.

Theoretical predictions are, however, difficult because the activation volumes of reaction steps and the compressibilities of SCFs change with pressure; a further complication is that, by changing the pressure, one simultaneously changes the density-dependent physically parameters of the SCF; effect on the mass transfer are also always present to some extent. Therefore, only apparent activation volumes have been measured for enzymatic reactions in SCFs. The reaction mechanisms of enzyme-catalyzed reactions are often not known.

Aaltonen[55] reported that apart from the direct conformational changes in enzymes, which may occur at very high pressures, pressure affects enzymatic reaction rates in SCFs in two ways. First, the reaction rate constant changes with pressure according to transition stage theory and standard thermodynamics. Theoretically, one can predict the effect of pressure on reaction rate if the reaction mechanism, the activation volumes and the compressibility factors are known. Second, the reaction rates may change with the density of SCFs because physical parameters, as the dielectric constant, change with density. These changes may indirectly influence enzyme activity.

The impact of operating conditions in the enzymatic esterification of *n*-octyl oleate catalysed by immobilized lipase from *Rhizomucor miehei*, was investigated.[56] The experimental evidence was that changing the pressure actually changed enzymatic reaction rate at constant substrate concentrations. The series of tests at various pressures were performed, in a constant volume reactor, keeping the substrate concentration constant. The results, presented in Figure 3, showed that in all the reported cases with the increase in pressure the reaction rate decreased. The reason of this particular behaviour could be explained by taking into account that with increase in the pressure, at constant volume, the molar fraction of substrates decreased. Consecutively, reduction in the initial reaction rate could be observed.

Moreover, investigations of *Mucor miehei*[57] lipase-catalysed esterifications in SC CO_2 showed a pressure increase from 10 MPa to 25 MPa reduced the initial reaction

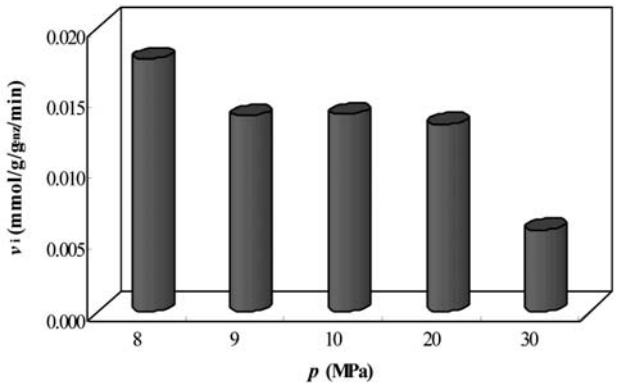

Figure 3: Effect of pressure on the initial reaction rate for the *Rhizomucor miehei* lipase-catalyzed esterification.

rates, paralleling the decreasing mole fraction of substrates. In Figure 3 it is evident that the results in initial reaction rate obtained in the range 9–20 MPa are approximately close: it seems reasonable that in this pressure range the substances solubility increase at higher pressures and at higher fluid-density is counter-balanced by the substrates mole fraction decrease. At 30 MPa the negative effect of low substrates mole fraction is predominant on the high and, anyway positive, substances solubility. On the other hand at 8 MPa, the solubility differs only marginally from the value at higher pressure (not shown). Moreover, it seems presumable that at higher pressure, a more considerable water amount was extracted from the enzyme beads, which resulted in lower reaction yields, in according with the investigation made by Knez et al.[58]

The effect of temperature was also investigated at various working pressures. The activity of *Rhizomucor miehei* lipase exhibits an optimum operating temperature for *n*-octyl oleate synthesis in the 70–80 °C range.[56] Due to a very wide optimal operative temperature range, even more at pressure level of 10 MPa, the curves reported in Figure 4 are typical for enzyme with high thermal stability

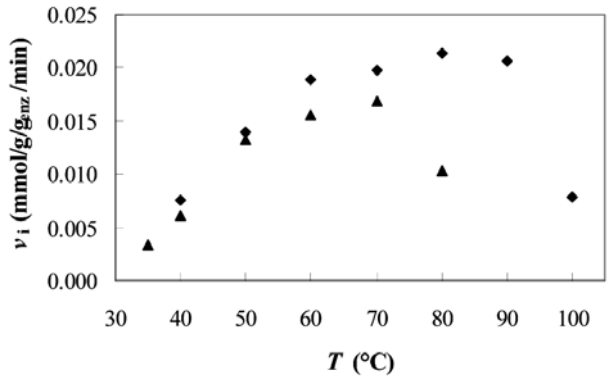

Figure 4: Combined effect of pressure and temperature on the initial rate for the *Rhizomucor miehei* lipase-catalyzed esterification: (●) 10 MPa; (■) 20 MPa.

and whose thermal denaturation, during the time of the assay, is negligible. At pressure level of 10 MPa an increase in temperature resulted also in higher solubility of substances in SCFs because the increase in the vapour-pressure of the compounds to be dissolved overcomes the reduction in density. In this case, the temperature effect, in addition to its effect on the enzyme activity, was directly related positively to the SCFs solvating power. Thus, operating in SCFs at 10 MPa a good compromise among optimum solvating power and enzyme activity, on one side, and enzyme thermal stability, on the other side could be found. At higher pressure, the negative temperature effect on the enzyme stability resulted predominant.

Modulating the pressure at different temperature, despite of important changes in the initial reaction rates, significant changes in the final ester contents were not observed. The results showed a relevant oleic acid conversion by octanol in *n*-octyl oleate up to 88% after 5 hours of bioconversion, obtained at various working pressures and optimal operational temperature of 70–80 °C. For each experiments-set at various pressures, at higher temperature a lighter decrease in final ester concentration was noted.

6. High-pressure Reactors

One of the main advantages of the use of dense gases as solvents for enzyme-catalyzed reactions is the simple downstream processing. The physico-chemical properties of dense gases which depend on the pressure and temperature are especially sensitive near the critical point. With changes in *p* and/or *T* less soluble substances may be forced to precipitate. Fractionation of the product and unreacted substrates is possible with step-by-step reduction of a dense gas solvent-power. Extraction of the mixture, usually with the same dense gas as used in reaction, but under different process conditions, allows fractionation, as well.

Several types of high-pressure enzymatic reactors have been used for the performance of enzymatic reactions in sub- and supercritical fluids: batch-, stirred-tank-, extractive semibatch-, recirculating batch-, semicontinuous flow-, continuous packed-bed-, and continuous membrane reactors.

Screening of enzymatic reactions in dense gases is usually performed in high-pressure batch-stirred-tank reactors. For continuous extraction of products from reaction mixtures (containing liquid substrates and an enzyme preparation), which shifts the reaction equilibrium towards formation of the product high-pressure extractive batch reactors are used. Kinetic studies of enzymatic reactions in dense gases may be performed in high-pressure recirculating batch reactors. With substrates saturated dense gas in the high-pressure semicontinuous-flow reactors is fed continuously through the enzyme bed. The abo-

ve-mentioned high-pressure reactors are well described in the literature.[55]

Each type of high-pressure reactors has its own advantages and disadvantages. In the high-pressure batch reactor the main disadvantage is expansion-induced deactivation in the case of enzyme reuse. In the high-pressure semicontinuous flow reactors the main disadvantage is that the concentration of substrates in dense gases cannot be varied, and that with changes of pressure and temperature, precipitation of substrates or products in the reactor can occur.

To avoid such disadvantages, a high-pressure continuous membrane reactor was designed. With respect to the low product specific catalyst costs, continuously operated biochemical systems are the most important from the industrial point of view.[59] For operating in SC conditions, appropriate membranes have to be used.

Two types of high-pressure membrane reactors have been developed lately: high-pressure continuous flat-shape membrane reactor and high-pressure continuous tubular-flow membrane reactor.

6. 1. Membrane Testing on Their High Pressure Stability

The decision about the proper material for membranes which could be used in the high pressure membrane reactor was made on the basis of their stability test in SC CO_2. Therefore membranes from different materials (cellulose acetate, polysulfon, nylon) were tested on their stability in SC CO_2.

Each membrane was treated with dry SC CO_2 and in the mixture of SC CO_2 and phosphate buffer solution (pH = 7).[60] After incubation, characteristics of the membrane, such as MWCO (molecular weight cut-off) and retention coefficient were determined and compared with the characteristics of the original membrane.

6. 2. Results of the Membranes-stability Test

Table 2 represents the results of the membranes-stability test. For the new polysulfon membrane with 10 000 MWCO the retention coefficient (R) was 92%. After the incubation in dry CO_2 the pore-diameter was enlarged. The R for PEG 10 000 dropt to 48% while for PEG 20 000 it was 79%. Probably dry CO_2 was the reason for larger pore diameter after the treatment.

To avoid this effect the membrane was treated with SC CO_2 and phosphate buffer (pH = 7). The R for PEG 10 000 dropt for 9% in this case and for PEG 15 000 it was 97%.

For nylon membrane the R for PEG 20 000 was 95%. After the treatment with dry CO_2 a small pore diameter increase was observed. R for PEG 20 000 after such treatment was 90%. After the treatment with CO_2 and phosphate buffer only minor changes in the membrane properties were observed; R in this case for PEG 20 000 was 93%. The change in retention coefficient in this case was only few (2) percent.

Results of those tests show that treatment of polysulfon or nylon membrane with combination of SC CO_2 and phosphate buffer does not influence on the characteristics of membranes.

6. 3. Enzyme-catalyzed Hydrolysis of Sunflower oil in a High-pressure Continuous Enzymatic Flat-shape Membrane Reactor (HP CEFSMR)

In the HP CEFSMR, the polysulfon or nylon membrane was used. The membrane in continuous high-pressure enzyme membrane reactor was placed between two sintered plates and fitted in the reactor, which was heated to constant temperature (Figure 5). The reactor was equipped with a magnetic stirrer. A certain amount of the lipase preparation was put in the reactor, while water, oil and the gas were separately pumped into the membrane reactor with the high-pressure pump, where biphasic medium appeared. One step reaction and separation unit was present in the system. The products and unreacted reactants were collected in the separator.

Application of flat-shape membranes in the high-pressure reaction system was studied on hydrolysis of

Table 2: Results of membranes – stability test in SC CO_2.

Material	Treated	Water flux (dm^3/h)	PEG (g/mol)	Hydrodynamic resistance R_h (kNh/m^3)	Retention coefficient R (%)
polysulfon	original	0.3167	10 000	3214	92
	SC CO_2	0.4784	10 000	2127	48
			20 000		79
	SC CO_2 + buffer	0.3509	10 000	2900	83
			15 000		97
nylon	original	0.3044	10 000	3343	66
			20 000		95
	SC CO_2	0.2975	20 000	3421	90
	SC CO_2 + buffer	0.2960	20 000	3438	93

Figure 5: High pressure continuous enzymatic flat-shape membrane reactor with sintered plates and flat membrane.

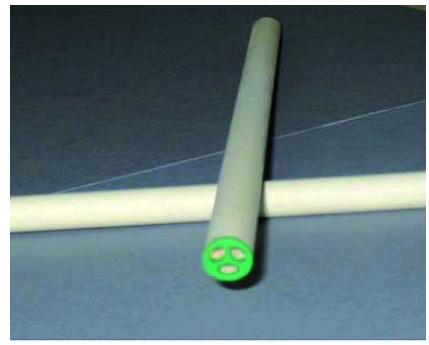

Figure 6: Ceramic membranes with high surface area used, as an enzyme carrier and as a separation unit, in the high-pressure membrane reactor.

sunflower oil in SC CO_2, catalyzed by non-immobilized lipase from *Aspergillus niger*. Experiments at different temperature and pressure combinations in SC CO_2 have shown that the highest concentrations of oleic and linoleic fatty acids were obtained at 50 °C and 20 MPa. Higher concentration of the product was achieved at higher temperature and pressure, because of the temperature activation of the enzyme and because of higher solubility of sunflower oil in SC CO_2. The solubility of sunflower oil in SC CO_2 at given reaction conditions was relatively low and therefore the process could be also limited by substrates solubility. The solubility of the products at higher temperature and pressure increased, which could normally affect the reaction rate.

The reactor continuously operated at the constant flow of both substrates till the conversion decreased significantly. Membrane was tested on its characteristics and it was almost unchanged in the comparison with characteristics of untreated membrane. Probably the enzyme deactivation was the reason for the decrease in conversion. After 70 h the change in total free fatty acid amount in the product was 40%.

6. 4. Enzyme-catalyzed Hydrolysis of Carboxy-methyl Cellulose (CMC) in a High-pressure Continuous Enzymatic Tubular Membrane Reactor (HP CETMR)

The HP CETMR was designed and constructed at the University of Maribor, Laboratory for Separation Processes and Product Design. For the immobilization of the enzyme on the ceramic tubular membrane covalent binding method over glutaraldehyde was used.[61] Tubular ceramic membranes with high surface area (Figure 6) were used as the enzyme carrier and as separation unit.

In spite of the different methodologies to attach enzyme molecules onto the membrane surface (covalent linkage, adsorption or electrostatic interactions, etc.), the maintenance of an appropriate hydrophilic microenvironment around the enzyme structure results in a clear improvement in stability towards water-stripping phenomena for processes in anhydrous media.[62] In this order, dynamic membranes formed by depositing water-soluble polymers onto a ceramic porous support exhibited excellent properties for continuous enzymatic processes in dry organic media. Also, the hydrophilicity of the gel layer formed onto the membrane surface presents a high permeability, and provided an adequate microenvironment to immobilize enzymes by covalent attachment (i.e. glutaraldehyde).[61]

The characterisation of the membrane before and after immobilization of the enzyme was done. The process of immobilization reduced the pore diameter for about 80%. This has to be taken in the consideration when the membrane for a defined process will be chosen.

Hydrolysis of CMC, catalyzed with covalent linked cellulose from *Humicola insolens* on the surface of ceramic membrane, was performed at atmospheric pressure and in SC CO_2 in the HP CETMR (Figure 7).

Reaction, carried out in SC CO_2 gave higher productivity then reaction, performed at atmospheric pressure. Hydrolysis of CMC in SC CO_2 and atmospheric pressure was performed for a long-term period. In both cases, the concentration of product slowly decreased with the time. The concentration of product after 46 hours of reaction in SC CO_2 was still higher than the concentration of reduced glucose achieved at reaction performed at atmospheric pressure.

The use of a membrane in the presence of the SCF improves the design of different attractive bioprocesses. This is dependent on the physico-chemical properties of SCFs and on the particular environment offered by membrane. The membrane reactors offer many advantages compared to the more traditional systems where the enzymatic reaction and the separation step take place separately.[63] If the reactor additionally operates continuous the advantages over batch-wise processes are expressed in easier process control, smaller amount of enzyme needed for reaction and also in production of the constant quality products.

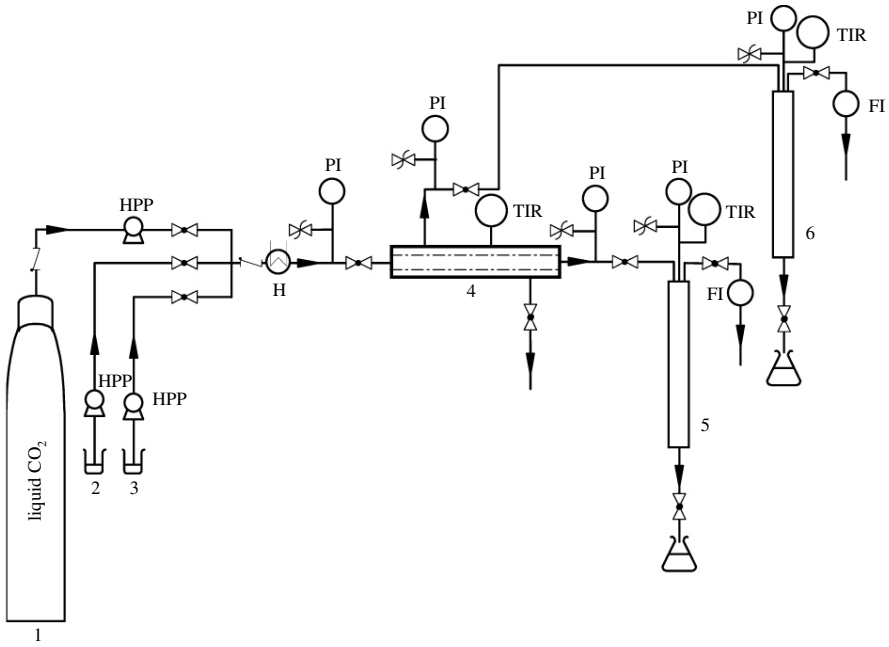

Figure 7: High-pressure continuous enzymatic tubular membrane reactor; 1 – gas, 2, 3 – substrates, 4 – membrane reactor with ceramic tubular membrane, 5, 6 – separation columns, HPP – high pressure pump; TIR – temperature regulator and indicator; P – pressure indicator; FI – flow indicator.

7. Conclusion

SCFs are promising media for enzymatic reactions. Their solvent power is easily tuneable and therefore they may be at the same time solvents for substrates and products. With their use only a single reaction/separation unit may be used to perform an enzyme-catalyzed reaction. An integrated extraction of products is very important when product inhibition appears.

The high pressure is usually not the reason for lower activity of biocatalysts when using SC CO_2 as a reaction medium. There are other reasons which cause the change in the enzymatic activity. On the other hand, in SC CO_2 an enhanced enantioselectivity of enzymes was observed.

However, because of the high operating costs, in the near future, SCFs will be used only for the production of high-valuable products.

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Povzetek

Encimi so lahko aktivni v različnih okoljih. Vodo, kot topilo *in vivo*, lahko delno ali v celoti nadomestimo z drugimi topili, kot so; mikro- emulzije, organska topila, reverzni miceliji, ionske tekočine in superkritični fluidi (SCFs). V SCFs so bile izvedene mnoge encimsko katalizirane reakcije. Vpliv SCF na stabilnost in aktivnost encima je v članku predstavljen na različnih primerih; z uporabo različnih reakcijskih sistemov (hidroliza, transesterifikacija ...), z uporabo prostih (*Subtilisin carlsberg, Aspergillus niger …*) in imobiliziranih encimov. Za izvedbo encimsko kataliziranih reakcij se uporabljajo naslednji tipi visokotlačnih encimskih reaktorjev; šaržni mešalni, ekstrakcijski polšaržni, recirkulacijski šaržni, polkontinuirani pretočni, kontinuirani z nasutim slojem biokatalizatorja in kontinuirani membranski reaktorji. Študije stabilnosti biokatalizatorja v visokotlačnem šaržnem mešalnem reaktorju so pokazale, da se aktivnost biokatalizatorja spreminja zaradi kompresije/dekompresije po vsaki šarži. Temu se lahko izognemo z uporabo visokotlačnega kontinuiranega membranskega reaktorja.