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Studies concerning lipase stability in batch-stirred-tank reactor under supercritical conditions

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Abstract

This study presents the factors affecting the enzyme stability in supercritical fluids (SC CO_2).

Immobilized lipase from Candida antarctica B, was incubated in SC CO_2 during 24 hours at different temperatures and pressure between 30° C… 50^oC, respectively, 10 … 20 MPa.

The enzyme was incubated in a high-pressure batch-stirred-tank reactor designed for operation at 300 bar and 200^oC.

After incubation and slow depressurization, the treated enzyme was used as biocatalyst for epoxidation reaction of sunflower oil. The results were compared with the conversion of reactions that was done with enzyme non-incubated in supercritical fluids.

Water content of biocatalyst incubated and non-incubated in SC CO_2 was determined by Karl-Fisher method.

Keywords: *Candida antarctica B,* enzyme stability, batch-stirred-tank reactor

Introduction

Many enzymes are stable and catalyze reactions in supercritical fluids, just as they do in other non- or micro-aqueous environments. Some of enzymes are more stable in supercritical fluids than in aqueous solutions.

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 [16].

The possibility of using supercritical fluids (SCFs) as «*tunable solvents*« not only for extraction (SFE – *supercritical fluid extraction*), but also for chemical synthesis (SFR – *supercritical fluid reaction*) is one of the many interesting features with their application in modern synthesis [16].

The advantages of SCFs associated with the use in chemical synthesis can be presented into four general categories:

- environmental benefits (do not contribute to smog, do not damage ozone layer, not acute eco-toxicity, no liquid waste);
- healthy and safety benefits (non-carcinogenic, non-toxic, non-flammable);
- process benefits (no solvent residues, facile separation of products, high diffusion rates, low viscosity, adjustable density, inexpensive);
- chemical benefits (high miscible with gases, variable dielectric constant, high compressibility, local density augmentation, altered cage strength).

Lipases gain much interest in chemical synthesis for formation and hydrolysis of esters, amides and peroxy carboxylic acids (peroxy acids or peracids) [17]. The catalytic action of lipases is stereospecific, so products that are obtained have high values, the enzyme is stable and can be reused.

Epoxized plant oils are used in PVC-plasticizers and in stabilizers field, as reactive dilutes for paints and for polyurethane-polyol production. Also peroxy carboxylic acids are important for bleaching industry and disinfection [10, 12].

The epoxidation reaction usually is catalyzed by a strong mineral acid (*Prileshajev method*), but also can be used lipase when the reaction takes under very mild conditions. An enzymatic peroxy acid formation is a safety and environmentally friendly alternative for chemical industry.

Considering the importance of the lipase from *Candida antarctica* B for many enzymatic reactions and the advantages of SCFs, the current study intended to stabilized the stability of enzyme under supercritical conditions. The enzyme incubated and non-incubated was used in chemo-enzymatic epoxidation of sunflower oil at atmospheric pressure.

Materials and methods

Enzymes and chemicals

Lipase (EC 3.1.1.3.) immobilized on a macro-porous anion exchange resin was a gift from Novo Nordisk A/S (Copenhagen, Denmark).

Sunflower oil and starch were supplied from supermarket Mercator (Slovenia).

Oleic acid with 99% purity, sodium hydroxide solution 0.1 mol/L, thiosulphate solution 0.25 $mol^{.}L^{-1}$, chloroform, iodine monochloride for synthesis, acetic acid 100% anhydre, toluene pro analysis, hydrogen peroxide 35% solution in water, hydrochloric acid fuming 37 % were purchased from Merck KDaA (Darmstadt, Germany). Potassium iodine powder pro analysis was supplied by Riedel-de Haen (Seelze, Gemany). Piridyn, phenophthalein and sodium hydrogen carbonate powder were obtained from Kemika (Zagreb, Croatia) and Merck (Darmstadt, Germany). Ethanol was supplied by Aldrich Chemical Co. (Diesenhofen, Germany).

Supercritical carbon dioxide (with 99.995% volume pure) was supplied by Messer MG (Ruše, Slovenia).

Determination of lipase thermal stability at atmospheric pressure

Lipase from *Candida antarctica B* was incubated at different temperatures at atmospheric pressure for 24 hours. After 24 hours of incubation, lipase was used for synthesis of epoxy acids.

Determination of lipase thermal stability in SC CO2

Lipase from *Candida antarctica B* was incubated in SC CO_2, in the high-pressure batch stirred-tank reactor at different temperatures for 24 hours. After slow depressurization, the pre-incubated enzyme was used as biocatalyst for epoxidation reaction of sunflower oil, at atmospheric pressure.

Synthesis of epoxy acids at atmospheric pressure

The epoxidation of sunflower oil, catalyzed by immobilized lipase (Novozym®435), was performed in a batch stirred tank reactor at atmospheric pressure. The reaction mixture

consisted of sunflower oil (7.8 g with 125 iodine value), oleic acid (0.56 g with 93.22 iodine value), lipase (0.8 g) and toluene(100 mL).[10,11,15]. The reactor was immersed in a water bath, heated to the desired operating temperatures and stirred by a magnetic stirrer (400 rpm).

When the operating temperatures was reached, the 35 % H_2O_2 (54 µl for 48 times, each portion after 7.5 minutes) was added to a mixture. Samples were taken from the reaction mixture at defined time periods and analyzed on iodine values. The reactions were performed for 50 h. After 50 hours, the enzyme was removed from the reaction mixture by vacuum filtration. The obtained product was washed with 20 ml distillated water to remove the excess of H_2O_2 and with 20 mL of 5 % $NaHCO_3$ solution in water, to remove the free fatty acids. The solvent (toluene) was removed by evaporation. [12].

The same epoxidation reactions, catalyzed by lipase incubated at atmospheric pressure and incubated in SC CO2 were performed, at the same conditions.

Assay procedure for determination of iodine value

Samples were taken from reaction mixtures at desired times and the iodine values was determined by AOCS procedures [18, 23]. Iodine value was obtained using the Wij´s method.

Results and discussions

Thermal stability of lipase from *Candida antarctica B* at atmospheric pressure

Thermal stability of Novozym®435 was tested by incubation at temperatures between 30 – 50°C at atmospheric pressure for 24 hours. After 24 hours of incubation, the pre-incubated lipase was used for synthesis of epoxy acids (in epoxidation reaction of sunflower oil) at atmospheric pressure and 40°C. The results obtained are illustrated in figure 1.

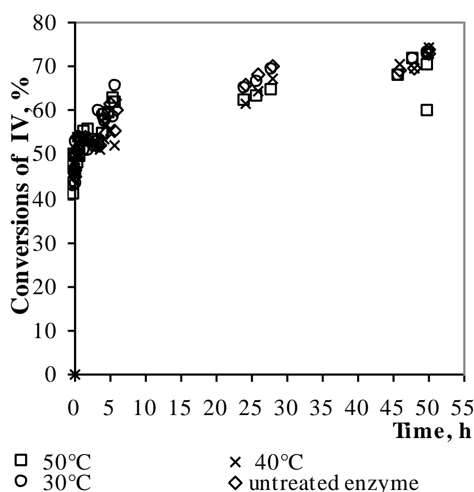

Figure 1. Conversion of IVs at 40°C and atmospheric pressure, catalyzed by untreated lipase and lipase that was exposed at different temperatures at atmospheric pressure for 24 h

The results show that there is no residual activity change in initial rates, the best conversion of iodine value was achieved with enzyme incubated at 40°C after 48 hours, 72 %, but in mean time, almost the same value was obtained with enzyme untreated, 74 %, respectively.

Thermal stability of lipase from *Candida antarctica B* in SC CO_2

To determine the stability of Novozym®435 in SC CO_2, lipase was incubated in the high-pressure batch stirred tank reactor in supercritical carbon dioxide for 24 h at different temperatures and pressure, between the range 30 – 50°C and 10 - 20 MPa, respectively. After incubation and slow depressurization, the pre-incubated enzyme was used as biocatalyst in the epoxidation reaction at 40°C and atmospheric pressure. For all the samples was determined the water content by the Karl-Fischer method.

Conversion of IVs obtained in epoxidation reaction at atmospheric pressure and 40°C with enzyme incubated at 10 MPa, respectively, 20 MPa and different temperatures are presented in figure 2 and figure 3.

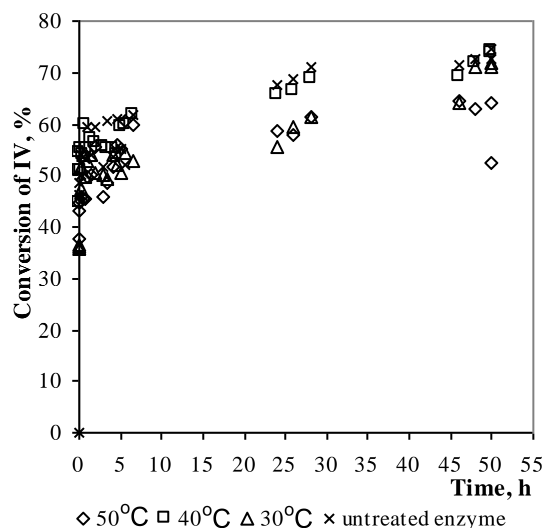

Figure 2. Conversion of IVs at 40°C and atmospheric pressure catalyzed by untreated lipase and lipase that was exposed to different temperatures in SC CO_2 at 10 MPa for 24 hours

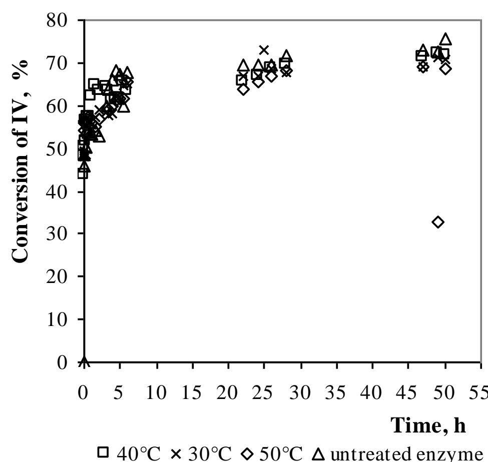

Figure 3. Conversion of IVs at 40°C and atmospheric pressure catalyzed by untreated lipase and lipase that was exposed to different temperatures in SC CO_2 at 20 MPa for 24 hours

Results showed that the conversion of iodine values decreased with incubation temperature from 30 – 50°C after 24 h of reaction performance. Decreased lipase residual activity at higher temperatures than 40°C can be explained by removing of essential water for enzyme for its vicinity. At higher temperatures water was extracted from the enzyme microenvironment by the SC CO_2.

These changes in activity between the crude enzyme preparation and the enzyme pre-incubated in SC CO_2 are connected with thermal activation/deactivation and with water distribution in the system.

Supercritical carbon dioxide may dissolve 0.3 – 0.5 % (w/w) water, depending by the pressure and temperature [4]. Untreated lipase contained 1.45% water, while the lipase which was previously exposed in SC CO_2 at 50°C and 10 MPa contained only 0.84% water. In mean time, for lipase exposed in SC CO_2 at 50°C and 20 MPa the water content was 0.97%, as it was measured by the Karl-Fisher method (Figure 4).

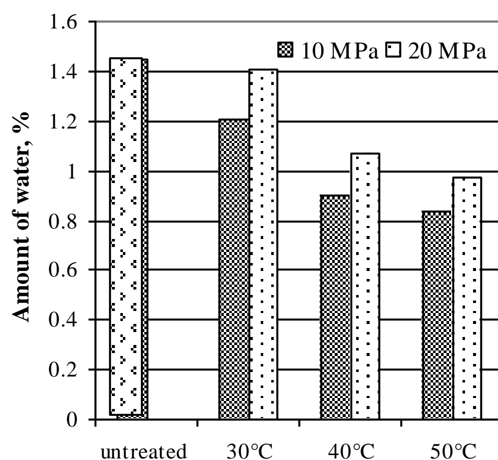

Figure 4. Water content in the lipase microenvironment after incubation (24 hours) in SC CO_2 at 10 MPa and 20 MPa and different temperatures

As can be see the amount of water is decreasing with increasing of temperature when the enzyme is incubated in SC CO_2 at 10 MPa and 20 MPa, respectively.
These results are in agreement with published results of activity of proteinase from *Carica papaya* latex treated with SC CO_2 at 30 MPa for 24 h, were the optimal temperature was 40°C [8].

Conclusions

In the literature is presented the thermal stability of others lipase from *Pseudomonas fluorescens*, *Rhizopus javanicus*, *Rhizopus niveus* in SC CO_2 and near-critical propane (30 MPa, 40°C) [8].

Immobilized lipase from *Candida antarctica B*, was incubated at atmospheric pressure and in SC CO_2 during 24 hours. After incubation the pre-treated lipase was used as biocatalyst in chemo-enzymatic epoxidation of sunflower oil. The reaction was carried out at atmospheric pressure. The higher conversion of IVs was achieved with enzyme incubated in SC CO_2 at 40°C and 10 MPa, which is recognized as a safe medium due to its non-toxicity and environmental benefits.

The thermal stability of *Candida antarctica B* lipase in SC CO_2 was higher than at atmospheric pressure. So, the lipase can be used as biocatalyst in SC CO_2 for hydrolysis of sunflower oil, for etherification of sugar fatty acids esters.

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