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ORIGINAL PAPER

Stability and activity of lipase in subcritical 1,1,1,2-tetrafluoroethane (R134a)

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Abstract The stability and activity of commercial immobilized lipase from *Candida antarctica* (Novozym 435) in subcritical 1,1,1,2-tetrafluoroethane (R134a) was investigated. The esterification of oleic acid with glycerol was studied as a model reaction in subcritical R134a and in solvent-free conditions. The results indicated that subcritical R134a treatment led to significant increase of activity of Novozym 435, and a maximum residual activity of 300% was measured at 4 MPa, 30 °C after 7 h incubation. No deactivation of Novozym 435 treated with subcritical R134a under different operation factors (pressure 2–8 MPa, temperature 30–60 °C, incubation time 1–12 h, water content 1:1, 1:2, 1:5 enzyme/water, depressurization rate 4 MPa/1 min, 4 MPa/30 min, 4 MPa/90 min) was observed. While the initial reaction rate was high in subcritical R134a, higher conversion was obtained in solvent-free conditions. Though the apparent conversion of the reaction is lower in subcritical R134a, it is more practicable, especially at low enzyme concentrations desired at commercial scales.

Keywords Activity · Stability · Lipase · R134a · Subcritical

Introduction

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) catalyze a variety of reactions. Although their natural reaction is hydrolysis of fats, they can be employed in microaqueous systems for esterification of fatty acids. Esters produced are, thus, suitable as flavors and fragrances in food. Esterification by lipases is beneficial because the flavor is considered natural by many food regulatory agencies [30]. However, enzymatic esterifications are heterogeneous and mass transfer limited and, hence, the rates are often too low to be of any commercial significance. This drawback has led to search for alternative media for enzymatic reactions to overcome the mass transfer limitations.

Since the first report on enzyme-catalyzed reactions in supercritical fluids (SCFs), defined as the fluids above their critical temperature and pressure [6, 12, 18, 23], much attention has been paid to the use of dense gases, mainly supercritical carbon dioxide, as potential alternatives to conventional organic solvents [1, 8, 14, 15, 20, 24, 27, 31]. The advantages of using supercritical fluids include the total replacement of organic solvent, coupled with higher diffusivity and lower viscosity that reduce interphase transport limitations, enhance the reaction kinetics, and tune reaction selectivity due to an appreciable increase in the local concentration of substrate and catalyst. Furthermore, the tunable solvating power of supercritical fluids facilitates the separation of reactants, products, and catalysts after reaction and, hence, the integration of biocatalytic and downstream processing steps in a single bioreactor.

Carbon dioxide is by far the most commonly employed supercritical solvent but solutes are limited to less-polar or highly volatile compounds, which means no proper dissolution of both hydrophobic and hydrophilic compounds. Furthermore, the hydrophilic characteristic of carbon dioxide over wide pressure ranges may affect negatively the activity of the enzyme, as the water partitioning between the enzyme and reaction mixture may be a key factor for conducting enzymatic reactions [19].

CO_2 is not the only gas whose critical properties fall in the range where biocatalysis is feasible, and not all alternative

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materials would be expected to chemically modify a protein. 1,1,1,2-tetrafluoroethane (R134a) is a relatively new gas that was introduced to replace the banned refrigerant R12. R134a is non-toxic, nonflammable, non-ozone-depleting and has a slight global warming capability [5]. The use of R134a as a solvent had been reported as "Phytosol A", an extraction process to recover natural products from a range of plant materials [28, 29]. R134a has a relatively low critical temperature and pressure (T_c = 101.21 °C, P_c = 4.059 MPa) yet is more polar (E_c = 5.0) than the chlorofluorocarbons (CFCs) previously studied and CO_2. Pressurized R134a is more hydrophobic than pressurized CO_2, although it seems hydrophilic being judged from other parameters such as permittivity and polarity parameter [13]. As indicated by many researches in which enzyme activity and stability in organic solvents increased with hydrophobic property (log*P*) of the solvents [16, 25, 26], pressurized R134a, with regard to its hydrophobic parameter, may be a promising medium for enzyme-catalyzed reactions.

Since about 20% of the total annual costs for running an enzymatically catalyzed chemical process that uses supercritical fluid as the only solvent are expenses for the biocatalyst [1], studies on enzyme stability and activity in SCFs are of great importance. Enzyme stability and activity may depend on the enzyme species, characteristics of compressed fluid, water content of the enzyme/support/reaction mixture and process variables manipulated. Inspection of the literature cited reveals that, while the relatively abundant data regarding activity and stability of enzymes in carbon dioxide, there is no corresponding experimental information for R134a.

Thus, the aim of this paper is to investigate the influence of pressure, temperature, exposure time and water content on the stability of a commercial immobilized lipase from *Candida antarctica* (Novozym 435), in subcritical R134a. Latter, the esterification of oleic acid with glycerol catalyzed by Novozym 435 was studied as a model reaction in subcritical R134a and in solvent-free conditions. The influence of pressure, temperature and water content on the activity of Novozym 435 submitted to subcritical R134a was investigated.

Materials and methods

Materials

Commercial immobilized lipase from *C. antarctica* (Novozym 435) immobilized on a macroporous anionic resin, was kindly supplied by Novozymes (Beijing, China). R134a (1,1,1,2-tetrafluoroethane) with a purity of 100% was product of Sinochem Modern Environmental Protection Chemicals Co., Ltd (Xi′ an, China). Other chemicals were from Sigma (St louis, USA).

Stability of lipase in subcritical R134a

A stainless steel high-pressure vessel has been used to perform the lipase stability tests (Fig. 1). Approximately, 0.5 g of Novozym 435 preparation was incubated with subcritical R134a under different conditions, pressure ranges from 2 to 8 MPa, temperature 30–60 °C, incubation time 1–12 h, water content 1:1,1:2,1:5 (lipase/water, w/w). After immediate depressurization (within 1 min), lipase activity was determined by the method described by Noel [21]. Additionally, to check the influence of depressurization rate on activity of Novozym 435 incubated in subcritical R134a, lipase preparation was incubated at 4 MPa, 40 °C in subcritical R134a for 7 h. After depressurization at different rates (within 1, 30, 90 min), lipase activity was determined. One unit of hydrolytic activity is defined as the amount of the enzyme that liberates 1 µmole equivalent of palmitic acid from tripalmitin in 1 min at 30 °C. Each value presented is the average of the at least three measurements. The stability criterion used was residual hydrolytic activity of the lipase incubated with subcritical R134a.

Esterification of oleic acid in subcritical R134a

The reaction was performed in a 275 ml stainless steel batch reactor (Fig. 1). The reactor immersed in a water bath of desired temperature, was first loaded with reactants, namely, 5.8 g of oleic acid, 2.8 g of glycerol, together with different amount of water, and then 0.12 g of Novozym 435 was added. R134a was compressed into the system up to a desired pressure, in the ranges 2–8 MPa. After the desired reaction time, the reactor was depressurized within 1 min and then R134a was liquefied and reclaimed. The reaction mixture was dissolved in 10 ml chloroform/methanol/water (2:1:0.6, v/v/v). The enzyme was settled by centrifugation

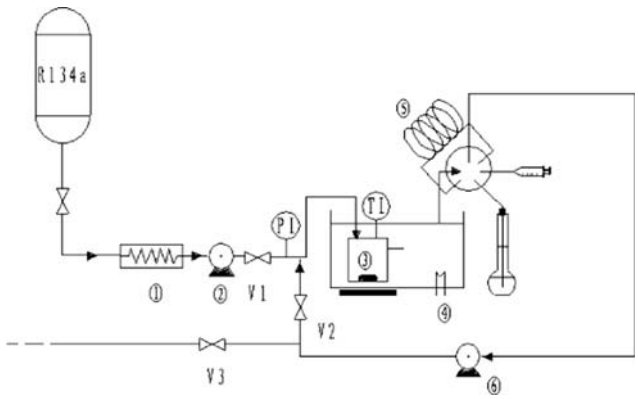

Fig. 1 Schematic illustration of the device used for enzyme stability and activity experiments, *1* cryostat, *2* piston pump, *3* high pressure vessel, *4* heater, *5* HPLC valve, *6* gear pump, *V1* inlet valve, *V2* valve for the cycle, *V3* outlet valve, *PI* pressure indicator, *TI* temperature indicator

and the lower phase was collected for analysis. The ester content was determined by calculating the residual oleic acid amount in the reaction mixture. The amount of oleic acid in the reaction mixture was measured using a volumetic method [11]. A mixture sample of 0.1 g (with an accuracy of 00.01 mg) was diluted in 20 ml of 0.1% (w/w) phenolphthalein solution in absolute ethanol and titrated with a standardized sodium hydroxide solution of 0.1 M in water.

Esterification of oleic acid in solvent-free system

Esterification of oleic acid was also performed in solvent-free conditions for comparison purpose, to investigate the effect of solvent property of subcritical R134a on the conversion as well as the activity of Novozym 435. Oleic acid (5.8 g), glycerol (2.8 g), together with 28 μm of water, and 0.12 g of Novozym 435 was added into a flask. The reaction was carried out at 40 °C, at 500 rpm, with a magnetic stir bar. After the desired reaction time, an aliquot sample (0.1 ml) from the reaction mixture was withdrawn and put into 5 ml chloroform/methanol/water (2:1:0.6, v/v/v). The solution was mixed well and centrifuged, and then the lower layer was collected for analysis.

Results and discussion

Stability of lipase in subcritical R134a

Pressure influence on stability of lipase

Figure 2 shows the residual activity of Novozym 435 after treatment with subcritical R134a under 2–8 MPa at 40 °C for 7 h. Incubation in subcritical R134a led to a significant increase of activity of Novozym 435, to a maximum value of 229% of the initial activity at 4 MPa. Within the range studied, no activity loss was observed and residual activity decreased with pressure increase above 4 MPa. This can be partially explained on the basis that R134a showed large changes in solvent properties over a relatively small pressure range, and its polarizability increased linearly with increasing pressure in subcritical region [2]. It has been argued that the decrease of lipase activity in supercritical fluids may be attributed to enzyme–solvent interactions. Therefore, the enzyme–solvent interactions change with pressure, which results in the variation of residual activity with pressure.

Debora de Oliveira et al. [22] reported that incubation in near-critical propane and *n*-butane led to increase of activity of Novozym 435, and by treatment with SC-CO_2, a decrease of enzyme activity was observed. However, that increase (up to 10%) was small compared to the results presented in this work.

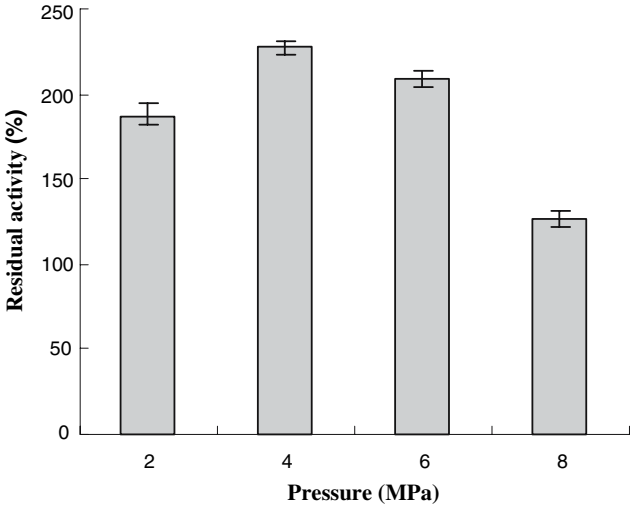

Fig. 2 Dependence of stability of lipase on pressure (40 °C, 7 h)

It should be emphasized that subcritical R134a was a relatively polar solvent and had higher solvent power for polar solutes even in subcritical domain (relatively lower pressure) than SC-CO_2, and this was of great interest for practical use of enzyme-catalyzed reactions. Then, 4 MPa was selected as working pressure in followed experiments.

Lipase thermal stability

As shown in Fig. 3, incubation of Novozym 435 at different temperatures presented excellent thermal stability. Incubated with subcritical R134a at 30–60 °C, an enzyme activity increase for several-fold (maximum of 300% of the initial activity at 30 °C) was observed. In contrast to SC-CO_2 treatment, which presented activity loss (5%) for Novozym 435 at 55 °C [26], no loss of enzyme activity was observed when Novozym 435 exposure to subcritical R134a, even at 60 °C. The extent to which enzymes show thermostability depends on the hydrophobicity of the media and the type of enzyme. For esterase from *Pseudomonas marginata*, lipase from *Candida rugosa* (Sigma), lipase from *Pseudomonas species* (Amano) and lipase AY from *Candida rugosa* (Amano) [10], only a decrease or no effect on enzyme activity was observed by treatment with SC-CO_2. Then, 40 °C was selected as working temperature for further experiments.

Incubation time influence on stability of lipase

In Fig. 4, the changes of activity of Novozym 435 after treatment with subcritical R134a for different times are illustrated. A time-dependent change of enzyme activity was observed. Even after 12 h incubation, the enzyme remained stable and active (residual activity, 199%). When

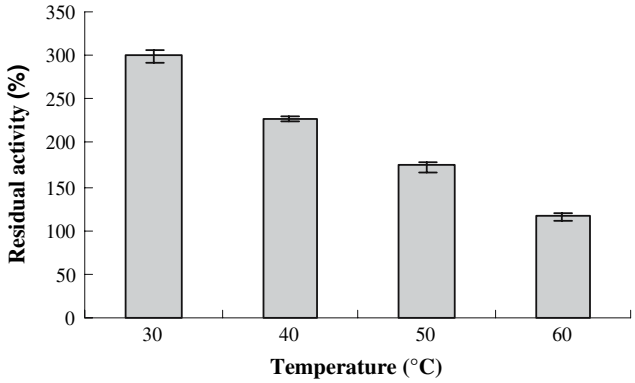

Fig. 3 Thermal stability of lipase (4 MPa, 7 h)

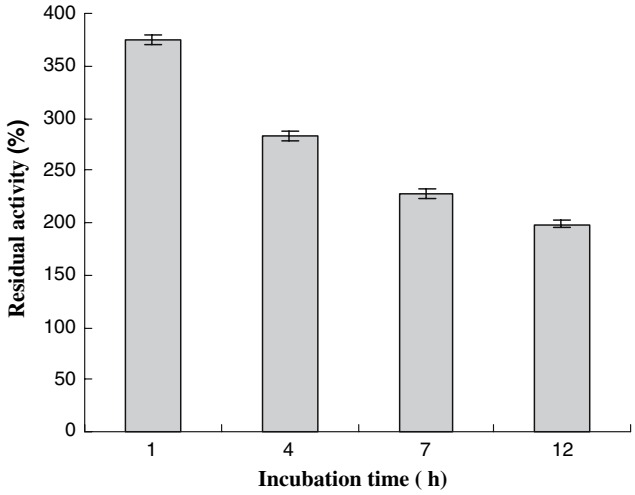

Fig. 4 Influence of incubation time on stability of lipase (4 MPa, 40°C)

considering the applicability of enzyme-catalyzed reaction in supercritical fluids, the enhanced reaction kinetics should be included. Reaction rates in supercritical fluids can be increased and satisfactory conversion achieved within several hours (always less than 6 h). The increased activity and good stability make it an attractive option to perform Novozym 435-catalyzed reactions in subcritical R134a.

Water content influence on stability of lipase

The stability of enzymes as well as their activity in supercritical fluid depends on the concentration of water [17]. Chulalaksananukul et al. [7] showed that the stability of immobilized lipase from *Mucor mihei* is always decreased after addition of water at temperature between 40 and 100 °C. The combined effects of high pressure, temperature and moisture content are even used to achieve inactivation of enzymes resulting in a lower seed quality [9]. The extent to which water content influences on stability of lipase depends on the hydrophobicity of the media, the immobilized supporter and the type of enzyme. Water is necessary for immobilized lipase to exhibit activity in supercritical fluids, and the amount of water adsorption to the immobilized supporter depends greatly on the solubility of water in supercritical fluids. The solubility of water in subcritical R134a is lower than that in SC-CO_2 [18] and as a result the distribution coefficient of water to the immobilized lipase in subcritical R134a is higher than that in SC-CO_2. Figure 5 showed the residual enzyme activities after subcritical R134a treatment at 4 MPa, 40 °C with increasing amounts of water for Novozym 435. As can be seen, activity increase for several-fold (maximum of 277% of the initial activity at 1:1, enzyme/water, w/w) was observed over a wide range. Novozym 435 is well suited for biocatalysis in subcritical R134a with production of high amounts of water.

Depressurization rate influence on activities of proteinases

To check the influence of depressurization rate on activity of Novozym 435 incubated in subcritical R134a, lipase preparation was incubated at 4 MPa, 40 °C in subcritical R134a for 7 h. After depressurization at different rates (within 1, 30, 90 min), lipase activity was determined. The residual activity of Novozym 435 was constant (227.8 ± 2.3%). These results indicate that depressurization rate has no effect on activity of Novozym 435. This can be explained since R134a is in subcritical domain (relatively lower pressure), without a liquid-supercritical phase transition.

Esterification of oleic acid with glycerol

Pressure effect

The effect of the pressure on enzyme activity was investigated varying the pressure between 2 and 8 MPa, at 40 °C, with 28 ul of water added. The results indicated a direct

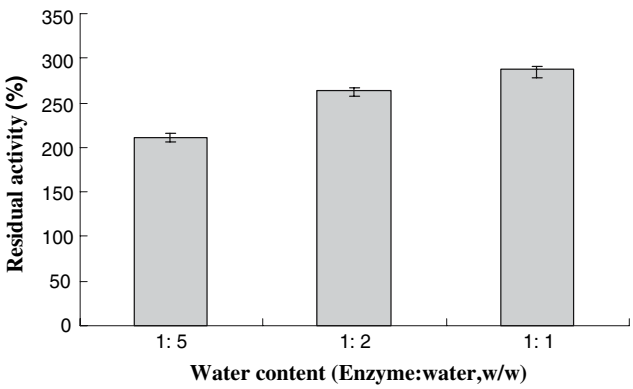

Fig. 5 Influence of water content on stability of lipase (4 MPa, 40 °C, 7 h)

dependence of conversion on the pressure. Reaction yield increased with pressure from 2 to 4 MPa, from 60.12% (5 h) to 70.24%, due to the enhanced mass transfer on the basis that rate-controlling step of glyceride formation in subcritical R134a was the diffusion of substrates from the liquid bulk through the boundary layer to the external surface of support. At 4 MPa the highest conversion was obtained, whereas at pressures above 4 MPa the conversion decreased to 63% (6 MPa) and 50% (8 MPa), due to the dilution effect of the substrates caused by larger R134a amount and lower relative activity under higher pressure.

Temperature effect

The investigation on the temperature effect was performed in the ranges 30–60 °C, at 4 MPa, with 28 µl of water added. The conversion was 60.11% (5 h) at 30 °C and increased to 70.24% at 40 °C. The conversion, however, dropped with further increase in temperature. Conversion of 58.24% was observed at 60 °C. The decrease in conversion may not be attributed to the deactivation of enzyme because residual activities of the enzyme were all above 100% in the explored ranges. This can be partially explained on the basis that higher temperature could shift the reaction equilibrium in favor of hydrolysis, lowering the yield of esterification. Additionally, lower relative activity at higher temperature may have an effect.

Effect of water content

The effect of the water on enzyme activity was investigated varying the amount of water added between 7 and 56 µl, at 40 °C, 4 MPa. A decrease in conversion with increasing water content was observed. The conversion with 7 µl of water added was 73% (5 h) and decreased to 66% with 56 µl of water added. As reported in previous works [3, 4, 32], enzyme needs a small amount of water to retain its active three-dimensional conformational state, even when the enzyme is covalently bound to a support. Since water is a product of the reaction, excess water would reverse the reaction and reduce the conversion of the acid.

Comparison of the esterification in subcritical R134a and in solvent-free conditions

A comparison with experimental results obtained in subcritical R134a and in solvent-free medium was conducted, to ascertain if the property of subcritical R134a plays an important role in the reaction performance. The conversion in solvent-free medium was 79.16% after 48 h, whereas in subcritical R134a at 4 MPa, 40 °C, the conversion was 73% after 5 h. The results indicated that higher velocity was attained in subcritical R134a due to substantial reduction in viscosity and enhancement in diffusivity of the reaction medium, minimizing the transport limitations towards the enzyme. It should be noted that the concentrations of substrates as well as enzyme were much higher in solvent-free medium because the total volume of the reactants is extremely small ($\leq$3.5 ml) unlike reaction in subcritical R134a where the reaction volume is 275 ml. Though the apparent conversion of the reaction is lower in subcritical R134a, it is more practicable, especially at low enzyme concentrations desired at commercial scales.

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