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The effect of water concentration on the activity and stability of CLECs in supercritical CO_2 in continuous operation

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Abstract

In this study the effect of the water concentration on a crystallized enzyme of *Candida antarctica* lipase B (ChiroCLEC™-CAB) in supercritical carbon dioxide ($scCO_2$) is studied. The model reaction used is the enantioselective esterification of racemic 1-phenyl ethanol with vinyl acetate; the reaction is performed in $scCO_2$ at 40 °C and 90 bar in batch and in continuous operation. Kinetic parameters have been derived from continuous experiments, leading to a catalytic turnover number of 0.95 s^{-1}. The optimum activity is reached at low water concentrations (0.05 g L^{-1}). At lower concentrations, CO_2 is stripping water from the enzyme leading to deactivation. However, adding a small amount of water to the substrates can reverse this deactivation and the enzyme activity is restored.

Keywords: *Candida antarctica* lipase B; ChiroCLEC™-CAB; Supercritical CO_2; Continuous reaction

1. Introduction

Lipases are a class of very stable enzymes that can convert a broad range of substrates, mainly in hydrolysis and esterification reactions [1]. *Candida antarctica* lipase B (Calb) is one of the most active and well-known lipases [2] used in various preparations [3]. Conventionally, enzymatic catalysis is performed in aqueous buffer systems at pH 7 and room temperature. In these systems, the enzyme is dissolved and most suitable to catalyze reactions involving hydrophilic components. About 20 years ago, however, Zaks and Klibanov [4] performed pioneering work concerning enzymatic catalysis in organic solvents. Moreover, it has been discovered that enzymes can maintain their activity in supercritical carbon dioxide ($scCO_2$) as well [5–7]. Changing the solvent from aqueous to organic has the advantage that more apolar substrates can be used, although immobilization of the enzyme to prevent denaturation is necessary [8,9].

For the use of these enzymes in supercritical fluids, several types of reactions have been performed. This has mainly been done in a batch mode, and only a limited number of studies have been performed (semi-) continuously, mainly in tubular and packed bed reactors [10,11]. In these studies mainly parameters like temperature and pressure have been varied [12–14]. Nevertheless, it can be anticipated that also the water activity in the system is one of the most important parameters for controlling the reaction rate [15]. One of the most widely used methods to control the water activity in a solvent is the addition of salt hydrates [16]. A disadvantage of this method is that salt hydrates can also interfere with the catalytic center of the enzyme and can therefore alter the catalytic activity not only by fixating the water activity [17,18]. Solid-state buffers can also be used to improve the activity, however, the effects of these buffers in $scCO_2$ are varying [19,20]. The most common technique for controlling the water activity in organic solvents involves the use of salt hydrates, whereas in $scCO_2$, this is usually done by controlling the ingoing water concentration [21–24].

Whereas most of the work described in the literature on the use of lipases in $scCO_2$ employs carrier bound enzyme, in this study cross-linked enzyme crystals of *C. antarctica* lipase B, ChiroCLEC™-CAB [25–28], have been used to catalyze reactions in $scCO_2$. The model reaction used is the enantioselective esterification of (*R*,*S*)-1-phenyl ethanol with vinyl acetate. The catalytic performance of the CLEC is evaluated both in batch and in continuous experiments with a focus on the effect of water concentration.

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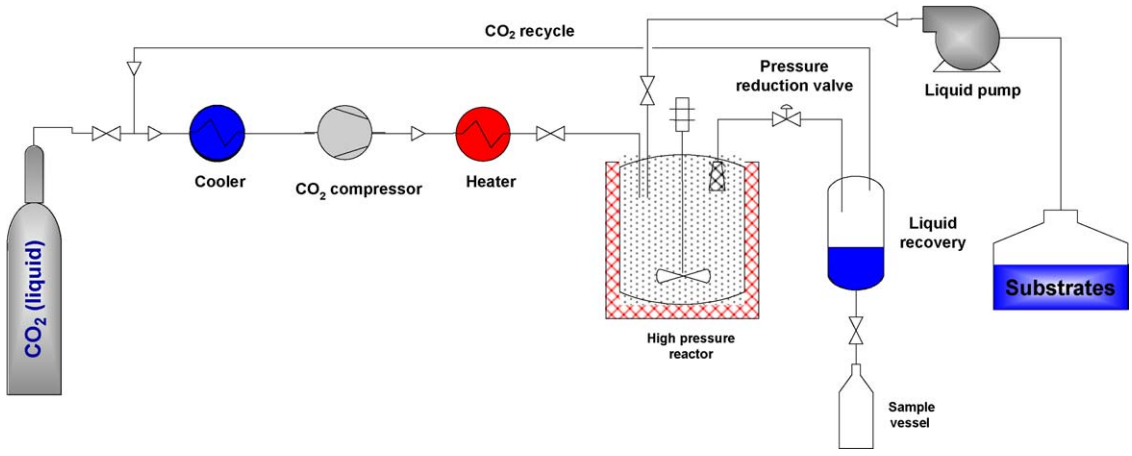

Fig. 1. Schematic high-pressure setup for the enzymatic reaction in supercritical CO_2 (with an optional CO_2-recycle).

2. Experimental

2.1. Batch experiments

Batch reactions in supercritical carbon dioxide were performed in a 100 mL stainless steel autoclave. The reactor was heated electrically (40 °C) and stirred with a magnetically coupled stirrer at 500 rpm. All batch experiments were carried out according to the following procedure. The reactor was filled with 10 mg of enzyme (ChiroCLEC^{TM}-CAB from Altus Biologics) and 10 mmol (*R*,*S*)-1-phenyl ethanol (Acros) and was heated until the desired temperature was reached. Because the enzyme can be sensitive to rapid changes in pressure, the reactor was slowly pressurized (at approximately 5 bar min^{-1}) by adding CO_2 (Hoekloos) until approximately 20 bar below the final reaction pressure. In the meantime, the reactant inlet was filled with 20 mmol vinyl acetate (Acros). The CO_2 required to reach the final reaction pressure (90 bar) was used to add the vinyl acetate to the reactor; this moment was taken as the starting point of the reaction. During the reaction, samples were taken with a 6-way HPLC valve with a 100-μL sample loop. The pressure in this sample loop was released by bubbling the solution through hexane. The sample loop was subsequently rinsed with hexane and the samples were analyzed with gas chromatography (GC) on an rtx-5 column with H_2 as the carrier gas.

2.2. Continuous experiments

The reactor setup that was used for the continuous experiments is shown in Fig. 1. The enzyme was added to a stirred stainless steel autoclave (200 mL) connected to a separator to recover the products. The reactor was slowly pressurized at approximately 5 bar min^{-1} and during the whole process the reactor was kept at 90 bar and 40 °C while stirring at about 500 rpm. In this setup the ChiroCLEC^{TM}-CAB catalyst was kept in the reactor using a suitable filter in the outlet of the reactor. Changing the total flow through the reactor varied the residence time in the reactor. Different water concentrations were obtained by adding salt hydrates, molecular sieves or water to the substrates before adding the substrates to the reaction vessel. The water content was measured by Karl Fischer titration. After the separator, samples were taken and were analyzed using GC.

In the continuous setup, three different amounts of enzyme were added to the reactor leading to final enzyme concentrations of 0.5, 1.0 and 5.0 mg mL^{-1}, respectively. The volumetric flow rate of the substrates was set to 10 vol.% of the total ingoing flow as this flow is adjusted with the pumps. This is in accordance with concentrations of 87 mM (*R*)-1-phenyl ethanol and 350 mM vinyl acetate in the reactor at 40 °C and 90 bar. The water concentration of the ingoing substrates (as measured with Karl Fischer titration) and the liquid CO_2 (60 ppm) lead to the calculated water concentration in the reactor.

3. Results and discussion

3.1. Comparison of batch and continuous experiments

The reaction appears to be completely enantioselective, therefore only the conversion of (*R*)-1-phenyl ethanol has been shown as a function of the substrate residence time in the continuous setup (Fig. 2). From this figure initial reaction rates can be derived, leading to values of about 1.8 mmol min^{-1} mg^{-1}

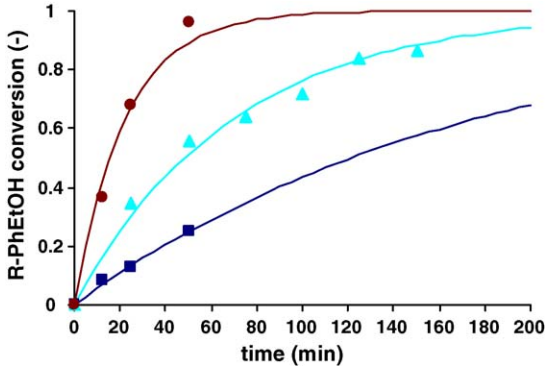

Fig. 2. Conversion of (*R*)-1-phenyl ethanol with vinyl acetate as a function of time. $P = 90$ bar, $T = 40$ °C. CLEC concentrations: (■) 0.5 mg mL^{-1}, (▲) 1.0 mg mL^{-1}, and (●) 5.0 mg mL^{-1}. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

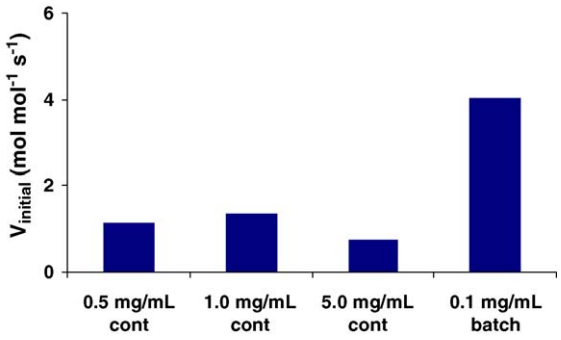

Fig. 3. Catalytic activity as a function of the CLEC concentration.

(Fig. 3). However, when these values are compared to the activity of the enzyme in batch, it appears that the activity in batch conditions is approximately a factor of 3 higher. As these values are expected to be the same, two improvements of the experimental procedure in the continuous setup have been explored.

To avoid accumulation of the enzyme in the O-ring closing the reactor, a catalyst basket is introduced to contain the enzyme. The catalyst basket prevents loosing the enzyme in the dead volumes of the reactor and is situated next to the stirrer. For organic solvents it has been reported that a substantial activity loss can occur in the first hour of operation when using CLECs [29,30]. Although the enzyme used in these studies is subtilisin Carlsberg and not *C. antarctica* lipase B it is likely that the deactivation is caused by the immobilization methods and not by the enzyme as such. Therefore, the 200 mL reactor has been extended with a sample loop making it possible to follow the conversion in time. A series of three experiments has been performed. First, a batch experiment is carried out in which the conversion is measured in time. Then, after full conversion is reached, the batch setup is changed into a continuous setup by continuously pumping CO_2 and substrates through the reactor at a fixed residence time. When steady state is reached, the situation is switched to batch again with the residence time of the continuous reaction as the starting point.

As can be seen from Fig. 4, the activity observed in the first batch reaction is initially lower than observed in the second one. Probably, transport of substrates into the catalyst basket is limiting. In this figure it is also shown that the activity at continuous conditions is not lower as compared to batch; the enzyme maintains its initial activity or becomes even slightly more active. Apparently, the inactivation within the first hour as observed in organic solvents does not occur in $scCO_2$.

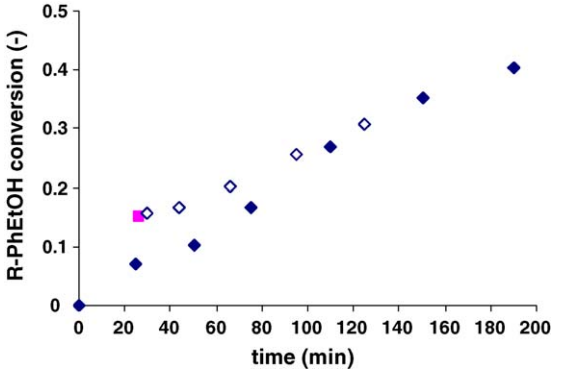

Fig. 4. Conversion of (*R*)-1-phenyl ethanol with vinyl acetate as a function of time with the use of a catalyst basket: (◆) first batch experiment, (■) continuous experiment, residence time is 23 min, and (◇) second batch experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

3.2. Kinetics

For this reaction, kinetics have been evaluated using a residence time of 13.2 min and an enzyme concentration of 0.5 mg mL^{-1} at a fixed vinyl acetate concentration of 350 mM and (*R*)-1-phenyl ethanol concentrations of 10.4, 21.2, 57.6 and 87.2 mM. From these data the initial velocities can be derived. Subsequently, the kinetic parameters are estimated assuming Michealis–Menten kinetics. It is known that *C. antarctica* lipase B shows ping-pong bi-bi kinetics, however, if one substrate concentration is kept constant it can be treated with pseudo-one-substrate kinetics [31], so that the equation for the kinetics is simplified to:

$$v_{ini} = \frac{V_m[\text{phenylethanol}]}{K_m + [\text{phenylethanol}]}$$

Non-linear regression in origin gives an apparent maximum velocity V_{max} of 0.013 (mM s^{-1}) and an apparent affinity constant K_m of 35.8 mM (Fig. 5). K_m is used to calculate whether external diffusion limitation occurs. The efficiency η is defined as

$$\eta = \frac{C_{si}(K_m + C_s)}{C_s(K_m + C_{si})}$$

in which C_s is the concentration of (*R*)-1-phenyl ethanol in the bulk phase, K_m the affinity constant and C_{si} is the (*R*)-1-phenyl ethanol concentration at the enzyme surface. With the method described by van't Riet and Tramper [32], C_{si} is obtained, yielding an efficiency of 0.999, indicating that no external diffusion limitation occurs. As it has been shown that diffusion in an enzyme crystal of lysozyme is slow [33], it is possible that internal diffusion limitation occurs. A general measure for the occurrence of internal diffusion limitation is the Thiele

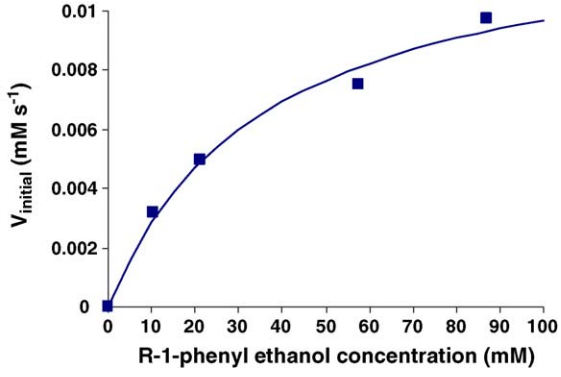

Fig. 5. Michaelis–Menten plot with fixed vinyl acetate concentration. $P = 90$ bar, $T = 40\,^{\circ}C$. The obtained parameters are $V_m = 0.013$ mM s^{-1} and $K_m = 35.8$ mM.

modulus:

$$\Phi = \sqrt{\frac{V_m R_p^2}{K_m D_{eff}}}$$

in which R_p is the radius of the enzyme crystal and D_{eff} is the effective diffusion coefficient of the substrate in the pores. The enzyme crystal has a diameter of 20 μm, and for the effective diffusion coefficient an estimation of 10^{-8} m^2 s^{-1} is made [34], yielding a Thiele modulus of 2×10^{-3}, indicating the absence of internal diffusion limitation. Moreover, the catalytic turnover number k_{cat} as estimated from the fitted V_{max} is around 0.95 s^{-1}, calculated with a molecular weight of 33 kDa of *C. antarctica* lipase B and the assumption of 90 wt.% of enzyme content in the crystal.

3.3. Water concentration

Since the water activity is one of the most important parameters to optimize the performance of the enzyme, it is useful to look at this effect. Although it would be more appropriate to consider the water activity instead of the water concentration, this quantity is practically less accessible, especially in (complex) supercritical mixtures. Therefore, the water concentration of the ingoing flow is adjusted with salt hydrates or molecular sieves. The effect of water concentrations between 0.05 and 2 g L^{-1} has been measured. The solid line in Fig. 6 gives the conversions at different water concentrations obtained for a fixed residence time of 13 min. Clearly the lowest water concentration (0.05 g L^{-1}) gives the highest activity, a value that gradually decreases with increasing water concentration. Reducing the amount of water present in the reactor even further by placing a fixed bed of silica in the CO_2 tubing shows a drastic decrease in activity (dashed line) indicating that at extremely dry conditions the enzyme is not able to keep the needed water molecules bound. The amount of water that is present in the reactor will distribute between the enzyme surface and the CO_2, resulting in net water stripping from the enzyme. This effect also occurs in polar organic solvents [35,36], where the establishment of the new water equilibrium appears to occur fast.

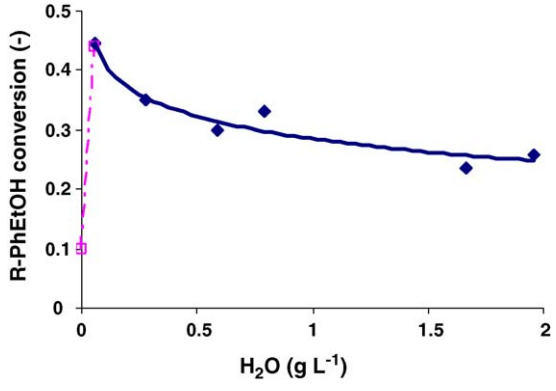

Fig. 6. Conversion of *R*-phenyl ethanol with vinyl acetate as function of the calculated water concentration in the reactor. CLEC concentration is 5 mg/mL, calculated residence time is 13 min. (◆) CO_2 with 60 ppm water is used and (□) CO_2 is dried over a silica column before use. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

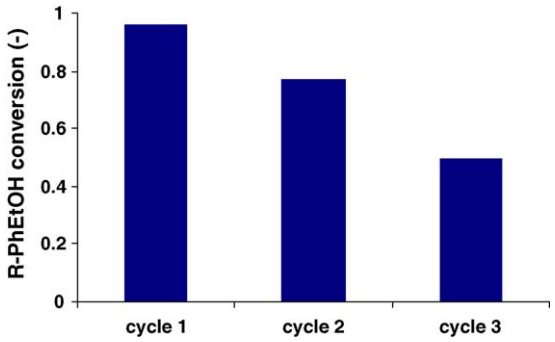

Fig. 7. Conversion of *R*-phenyl ethanol with vinyl acetate after treatment with CO_2. After each cycle the reactor is flushed for 60 min with pure CO_2 at 20 mL min^{-1}. The residence time is fixed at 25 min.

3.4. Influence of CO_2 flow

Since the water concentration in the reactor is dependent on the continuous in and outflow of CO_2 and reactants, this allows for a relatively easy control. As shown in the previous paragraph, an optimum in water concentration can be found. However, by decreasing the amount of water even more, the essential water molecules are pulled away from the enzyme causing a lower activity. The gradual effect of stripping the water by CO_2 can also be illustrated by varying the CO_2 flow.

Experiments have been performed in which a continuous reaction has been run for 2 h (cycle 1). After this cycle, the reactor is flushed for 60 min with a high flow of CO_2 (20 mL min^{-1}). After this, the conversion is measured after two hours at the same fixed residence time again (cycle 2). This step is repeated once more (cycle 3). As it can be seen in Fig. 7, the decrease in activity is tremendous: from 96% conversion in cycle 1 to only 50% conversion in cycle 3. Interestingly, this effect appears to be rather reversible (Fig. 8). Since the division of water molecules between the enzyme and CO_2 is a rather rapid process, constant activities can be reached by continuously pumping CO_2 and substrates with constant water concentrations through the reactor.

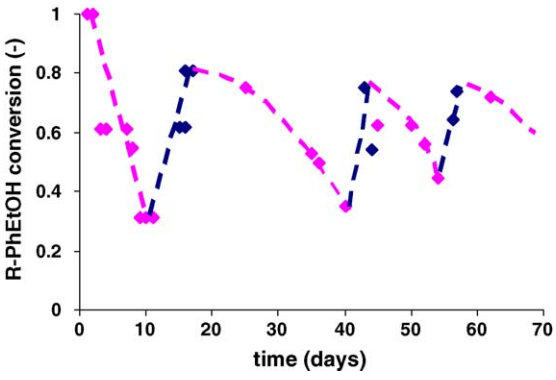

Fig. 8. Conversion of *R*-phenyl ethanol with vinyl acetate as a function of number of days on stream for continuous measurements. (◆) No addition of water and (◆) after addition of water. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

In this way optimal conditions can be found. In aqueous solutions the hydration state of the enzyme has been measured with FTIR, from which it has been concluded that lipase can bind up to 660 water molecules, a value corresponding to three or four hydration monolayers [37]. In CO_2, however, the release of the CO_2 pressure to atmospheric pressure will pull the water away from the enzyme, therefore, the hydration level of the enzyme in optimal supercritical CO_2 conditions cannot be measured.

3.5. Stability

As shown above, the activity of *C. antarctica* lipase B in the crystallized form is proven to be high. Even though the activity of the enzyme decreases when rinsed with CO_2, this decrease can be reversed by the addition of water as shown in Fig. 8. The same batch of enzyme has been used for more than two months. As dry CO_2 has been used in this experiment, the activity decreased within 9 days to about one third of the initial value. Subsequently, 0.5 wt.% water has been added to the substrates and the activity of the enzyme increased again to about 80% of the initial activity, a procedure that could be repeated several times without any additional loss of activity. This underlines the importance of controlling the water concentration in $scCO_2$, especially in continuous operation.

4. Conclusions

Cross-linked enzyme crystals of *C. antarctica* lipase B appear to be very stable enzyme preparations, showing a high activity in supercritical CO_2 with a turnover number of 0.95 s^{-1}. It is shown that CO_2 strips the necessary water from the enzyme leading to a decreased activity. However, this effect can be reversed by the addition of water to the system leading to an immediate increase in activity again.

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