ENZYMATIC CATALYSIS IN $scCO_2$ FOR KINETIC RESOLUTION OF CHIRAL ALCOHOLS IN BATCH, CONTINUOUS AND SEMI CONTINUOUS MODE

H. Weyten*, Z.Dijkstra, J.T.F. Keurentjes, L. Willems, K. Elst, L. Van Ginneken
Vito, Environmental and Process Technology, Boeretang 200, B-2400 mol, Belgium,
herman.weyten@vito.be; fax: +32 14 32 11 86

Synthesis of enantiomerically pure compounds are becoming more and more important in pharmaceutical, agrochemical and fine chemical industry. The use of enzymes as enantioselective catalysts in kinetic resolution reactions is one of the most useful methods to obtain these pure enantiomers from racemic mixtures because enzymes can exhibit extreme high selectivity and yield. In this study a crystallized enzyme of Candida Antarctica lipase B (ChiroCLEC^{TM}-CAB) is used in $scCO_2$ –being a green environmentally friendly solvent– in a model esterification reaction of ±1-phenyl ethanol (1-PhEtOH) with vinyl acetate (AcOVi). In all tested conditions, the enantioselectivity of the enzyme exceeds 99%. Initial reaction rates in $scCO_2$ are up to three times higher than in a conventional organic solvent (hexane). The enzyme, however, was found to be sensitive to large and/or sudden pressure differences, making reuse of the catalyst in batch processing very difficult. In a (semi) continuous reactor setup the ChiroCLEC^{TM}-CAB catalyst is kept in the reactor –at the desired process temperature and pressure– by using a suitable filter in the outlet of the reactor. The process was run, at the same conditions for a period of over 2 weeks without changing that catalyst at 40°C and 90 bar both in continuous and semi continuous mode. Over this period of time the activity dropped to about 30% of its original value. This deactivation process can however be reversed by adding a small amount of water to the reaction mixture. This indicates that during continuous and semi continuous processing, the (very) small amount of water –which is necessary for the enzyme to be active– is 'stripped' from the catalyst. However, adding a small amount of water regenerates the catalyst.

I - INTRODUCTION

Traditionally, enzymatic reactions were performed in aqueous solutions but in the early eighties it was demonstrated that enzymes are also active in organic solvents [1, 2], which increases the potential applications for enzymatic catalysts tremendously. Because environmental aspects are becoming more and more important in the process industry, supercritical CO_2 ($scCO_2$) –being a green and GRAS (generally regarded as safe) solvent– will gain popularity over many traditional solvents [3]. The high diffusivity and low viscosity of $scCO_2$ offer great opportunities to overcome diffusion limitation in chemical reactions. Additionally, physical and chemical properties of $scCO_2$ can be modified simply by changing temperature and/or pressure, which makes it possible to tune the solvent properties to match the proper reaction conditions.
The use of enantiomerically pure components in the agrochemical, pharmaceutical and food industries will become even more important in the near future [4]. Enzymes are known to be very selective biocatalysts, exhibiting a very high enantioselectivity for reactions with racemic mixtures. Combining enzymatic catalysis with the 'green' $scCO_2$ solvent offers great potential for environmentally friendly synthesis of enantiomerically pure components or separation of enantiomerically pure products from racemic mixtures by kinetic resolution [5, 6]. Enantioselective enzymes can, however be relatively expensive. Therefore, in order for the

process to be economical it is necessary that the catalyst is recycled without loss of activity. In this study a continuous and semi continuous process was studied for a model esterification reaction in $scCO_2$. The performance of the process is compared to simple batch experiments.

II – MATERIALS AND METHODS

The model reaction is the enantioselective esterification of ±1-phenyl ethanol with vinyl acetate (figure 1) using the Candida Antarctica lipase B. The enzyme is used as a cross-linked enzyme crystal (ChiroCLEC^{TM}-CAB) from Altus Biologics which is reported to have higher stability and activity compared to the 'free' enzyme [7]. Before use, the CLEC CAB enzyme was washed with tert-amyl alcohol and hexane for at least three times.

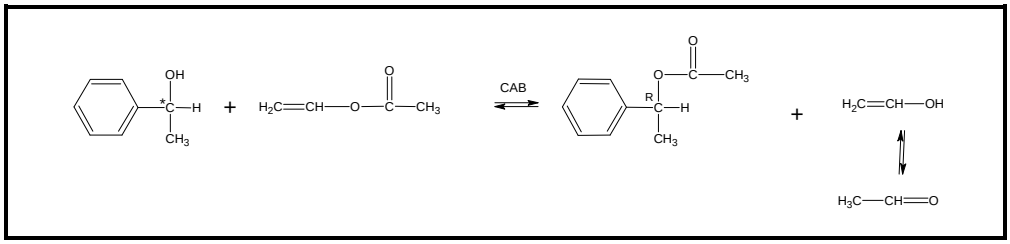

Figure 1: Model reaction of ± 1-phenyl ethanol (1-PhEtOH) with vinyl acetate (AcOVi).

The reaction in hexane at atmospheric pressure was used as a reference. Batch experiments in $scCO_2$ were performed in 100 ml stirred autoclaves at 40°C. Conversion, selectivity and reaction kinetics –assuming simple Michaelis-Menten kinetics– were studied at different process conditions: pressures, concentrations, and ± phenyl ethanol ÷ vinyl acetate ratios. Conversion and selectivity were measured using gas chromatography (GC) and were compared to the novozyme 435 enzyme.

The reactor setup that was used for the (semi) continuous experiments is shown in figure 2. The CLEC CAB catalyst (1mg/ml) is loaded in a stirred (200 ml) autoclave. Because the enzyme is sensitive to large and or sudden pressure drops, the reactor is pressurized very slowly (> 4 hours) up to 90 bar and is heated to 40°C. The enzyme is kept in the reactor by using a suitable filter in the outlet of the reactor, which is kept at the same conditions during the whole process while stirring at ~500 rpm.

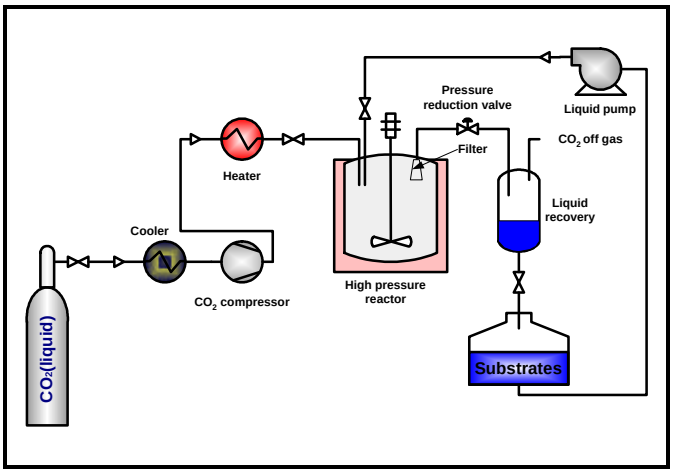

Figure 2: Schematic diagram of continuous reactor setup.

In the continuous and semi continuous experiments, ±1-PhEtOH ÷ AcOVi ratio was fixed at 1 ÷ 2 mol/mol. In the continuous experiments the residence time in the reactor was varied by changing the total flow through the reactor. The liquid (±1-PhEtOH + AcOVi) concentration in the $scCO_2$ during injection was fixed at 10 Wt%.
In order to obtain a more than 75% conversion, the residence time needs to be ~ 200 minutes. In order to reach these residence times in the setup that is used, very low flow rates have to be used, which makes it difficult to control the pressure accurately. Therefore in the second set of experiments (semi continuous process) the total flow rate was fixed at 7.1 g/min using the same concentrations of 1-PhEtOH, AcOVi and CO_2. The solution was reinjected several times to obtain higher residence times.

III – RESULTS AND DISCUSSION

III.A – Batch experiments

In a previous study [8] catalyst loading, ±1-PhEtOH and AcOVi concentrations were optimized taking into account the solubility of the reactants and products in $scCO_2$. It was reported that the ChiroCLEC^{TM}-CAB enzyme is very selective for the reaction of R-1-PhEtOH reaction resulting in an enantiomer excess ($ee_R = (C_R–C_S)/(C_R+C_S)$) of over 99%. The ChiroCLEC^{TM}-CAB enzyme is much more active for this esterification reaction than the novozyme 435 enzyme (figure 3a). In hexane, about 10 times more novozyme 435 is needed to obtain the same conversion compared to the cross-linked enzyme crystal. This lower activity may be caused by the reaction of acetaldehyde –a by product of the esterification reaction (figure 1)– with free amine groups in the novozyme enzyme and/or carbamate formation (reaction of CO_2 with the free amine groups), which –in the cross-linked form– may not be available for reaction. Initial reaction rates –assuming simple Michaelis-Menten kinetics– indicate about 3 times higher activity of CLEC CAB in $scCO_2$ compared to hexane (~0.24 mol/h g). However, the activity decreases with increasing pressure. At ~125 bar the activity in $scCO_2$ (ρ= 685 kg/m³) is about the same as in hexane which, (at 40°C) has about the same density (figure 3b). Although the ChiroCLEC^{TM}-CAB enzyme is active and stable (at least over a period of more than 4 hours) in $scCO_2$, it was observed that activity is reduced with large and/or sudden pressure differences.

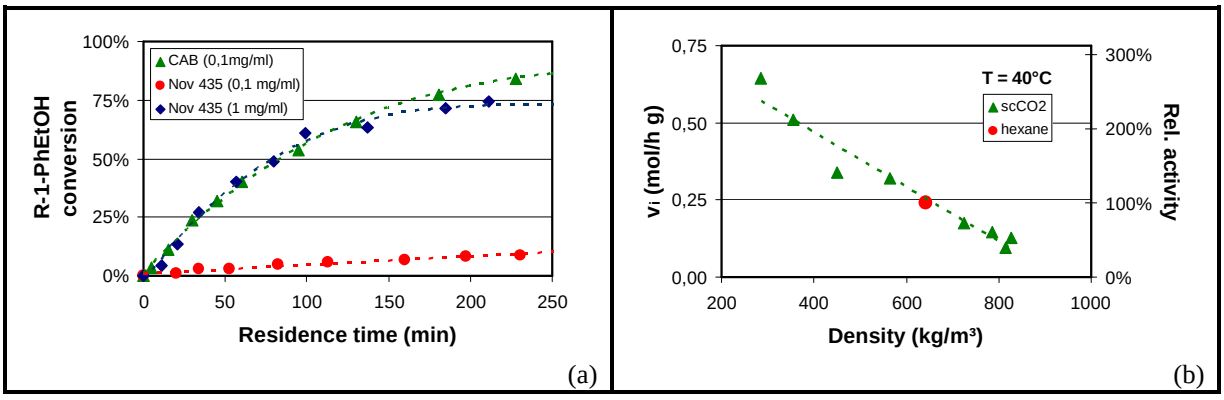

Figure 3 a: Conversion at 40°C of R-1-PhEtOH in hexane vs. residence time for ChiroCLEC^{TM}-CAB enzyme (0.1 mg/ml) compared to the 'free' novozyme 435 (0.1 mg/ml and 1 mg/ml). 1-PhEtOH ÷ AcOVi=1÷2 mol/mol.
b: Initial reaction rate for ChiroCLEC^{TM}-CAB enzyme (0.1 mg/ml) reaction of 1-PhEtOH and relative activity –compared to the reaction in hexane– in $scCO_2$ at different CO_2 densities (different pressures at 40°C).

III.B – Continuous and semi continuous experiments

Continuous experiments were run over a period of more than 2 weeks using a ±1-PhEtOH ÷ AcOVi = 1 ÷ 2 mol/mol in $scCO_2$ at 40°C and 90 bar. The concentration of the reactants in the $scCO_2$ during injection was 10 Wt%. For every run 25 ml of the reaction mixture was used. By determining the R-1-PhEtOH conversion for different (average) residence times in the reactor, the reaction rate was calculated assuming simple 1st order kinetics and were compared to the reaction in hexane (= set at 100%). It was observed that after every experiment catalyst activity decreased (● in figure 4b) indicating that the ChiroCLEC™-CAB enzyme deactivates when used over a longer period of time in $scCO_2$. It was believed that this was due to pressure fluctuations that were caused by difficulties keeping the pressure stable at 90 bar when using the very small flows (total of 1 to ~4 g/min) that are necessary to obtain residence times which give reasonable conversions.
The semi continuous experiments (starting with fresh catalyst) were done using the same conditions. However, a total flow (CO_2+PhEtOH+AcOVi) of 7.5 g/min was used, which made accurate pressure control much easier. For each run 40 ml of reaction mixture was used and was reinjected several times to obtain the higher average residence times. Although pressure variations during processing were less than 5%, deactivation of the ChiroCLEC™-CAB enzyme was also observed (figure 4a and ■ in figure 4b). The reaction rate constant dropped to about 30% of its original value over a period of 11 days.
However, when a small amount (0.5%) of water was added to the reaction mixture the ChiroCLEC™-CAB enzyme could be reactivated (◆ in figure 4b). Although previous studies indicated that the ChiroCLEC™-CAB catalyst activity is almost independent of the water activity for the reaction that was studied, it is a well known fact that enzymes need a (very) small amount of water to be able to function. The fact that the deactivated catalyst can be reactivated by adding a small amount of water, indicates that during continuous and semi continuous processing, water is 'stripped' from the catalyst.

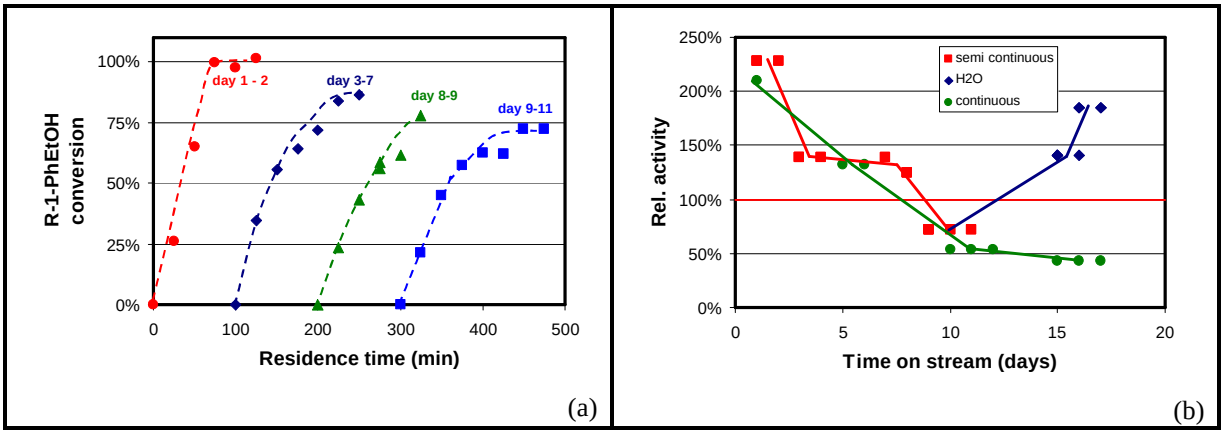

Figure 4 a: Conversion of R-1-PhEtOH in $scCO_2$ (at 40°C and 90 bar) vs. residence time for ChiroCLEC™-CAB enzyme in semi continuous experiments. (Different curves are shifted over 100 minutes over the X-axis). Number of days on stream is also indicated.
b: Relative activity (compared to reaction in hexane) for ChiroCLEC™-CAB enzyme as function of number of days on stream for continuous and semi continuous measurements.

IV - CONCLUSIONS

The experiments have shown that ChiroCLEC^{TM}-CAB (cross-linked enzyme crystal of Candida Antarctica lipase B) is a highly selective and active biocatalyst for the enantioselective esterification of ±1-phenylethanol with vinyl acetate. It is superior to the 'free' novozyme 435 enzyme for this reaction. It is about 3 times more active in scCO_2 than in hexane at low pressure (80 bar), but the initial reaction rate in scCO_2 becomes comparable to the value observed in hexane when the CO_2 density is about equal to the density of hexane at the same temperature. The enzyme was observed to be sensitive to large and/or sudden pressure differences, making reuse of the catalyst in batch processing very difficult.
A reactor setup was developed in which the ChiroCLEC^{TM}-CAB catalyst is kept in the reactor by using a suitable filter in the outlet of the reactor. This makes it possible to run the process (semi) continuously while the enzymatic catalyst can be kept at the required temperature and pressure. The process was run at the same conditions for a total period of over 2 weeks without changing that catalyst both in continuous and semi continuous mode at 40°C and 90 bar.
Although pressure variations during processing were very limited, deactivation of the ChiroCLEC^{TM}-CAB enzyme was observed. The activity dropped to about 30% of its original value over a period of running experiments for 11 days. During continuous and semi continuous processing, the (very) small amount of water –which is necessary for the enzyme to be active– is 'stripped' from the catalyst. This deactivation process can, however, be reversed by adding a small amount of water to the reaction mixture, which regenerates the catalyst. Further experiments using the same catalyst are still in progress and detailed kinetic studies are being done.

REFERENCES

[1] ZAKS A., KLIBANOV A.M.; Science, **1984**, p 1249.
[2] NAKAMURA K.; Trends in Biotechnology, Vol 8, **1998**, p 288.
[3] ACS; "New process technology", ACS publication, July **2001**
[4] ROUHI A. M., Chem. Eng. News, Vol 81 (18), **2003**, p 45.
[5] KAMAT S.V., BECKMAN E.J., RUSSELL, A.J., Critical Rev Biotechnology, Vol 15, **1995**, p 41
[6] MESIANO A.J., BECKMAN E.J., RUSSELL, A.J., Chemical Reviews, Vol 99, **1999**, p 623
[7] MARGOLIN A.L., Trends in Biotechnology, Vol 14(7), **1996**, p 223
[8] DIJKSTRA Z.J., WEYTEN H., KEURENTJES J.T.F., proc. ECCE-4, Granada, Vol 11, **2003**, 12.1-002