ON SILICA GELS IMMOBILIZED CELLULASE AS BIOCATALYST FOR HYDROLYSIS OF CARBOXY-METHYL CELLULOSE

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Cellulase preparation Celluzyme 0.7T was immobilized on silica gels using standard sol-gel procedure. To compare the properties and activity of immobilized enzymes, gels were dried by two different drying routes: conventionally in the air at 40°C (xerogels) and supercritically with CO_2 at 40°C and 100 bar (aerogels).
The properties of enzyme preparations in the form of aerogel and xerogel were compared at atmospheric pressure and at the same time with the properties of crude enzyme preparation.
Study of cellulase preparation (Celluzyme 0.7T) activity in three different forms in SC CO_2 will be reported.
The aerogel and xerogel cellulase preparations were successfully reused many times in the high-pressure batch system.

INTRODUCTION

Enzyme-catalyzed reactions are superior to conventional chemical methods due to mild reaction conditions, high catalytic efficiency and the inherent selectivity of natural catalyst, which results in much purer products and lower energy input. A milestone in the use of enzymes as catalysts for organic chemistry was the discovery that some enzymes retain their catalytic activity in non-aqueous media [1]. Supercritical fluids (SCF) are a unique class of non-aqueous media with many features (due to their physico-chemical properties) that make their use as a solvent for biocatalysis and separation particularly desirable [2]. Supercritical media retain good enzyme activity and stability [3]. An additional benefit of using enzymes in supercritical fluids is that it provides a very convenient way for the recovery of products or recovery of non-reacted components. The most useful temperature range of SC CO_2 and the typical, low operation temperatures of enzymes ideally overlap each other.
Immobilization of the enzyme on a solid support is one of the most widely employed methods for using it in continuous operated packed beds and stirred tank reactors, especially for large scale operation; it is usually very easy to separate the enzyme from the product solution. Since enzymes are normally not soluble in organic or supercritical media there is no need for covalent linkages between the support and the enzyme. It is advantageous to use a porous support so that the enzyme is spread on a large surface area.
Aerogels are advanced materials that consist normally of more than 96% air, while the remaining 4% is a matrix of silicon dioxide. Aerogel, consequently, is one of the lightest solids ever conceived and it has unique properties such as high porosity, large surface area, low density and low thermal conductivity.

Due to these remarkable properties aerogels could serve as good carriers for metal catalysts as well as for biocatalysts.
In the present work application of a sol-gel encapsulation technique for the immobilization of cellulase into silica aerogel and xerogel matrices have been studied. The so immobilized enzyme was used as biocatalyst for hydrolysis of carboxymethyl-cellulose at atmospheric pressure and in SC CO_2. Results were compared with the results, obtained at the use of crude enzyme preparation, as biocatalyst for the same reaction.

I – MATERIALS AND METHODS

Chemicals:

Cellulase from fungus *Humicola Insolens* (Celluzyme 0.7T) was kindly donated from NOVO Nordisk A/S, Denmark. Tetramethoxysilan (TMOS) was used as a commercial product from Aldrich (purity 98%). Carboxymethyl-cellulose (C-4146), 3,5 dinitrosalycic acid (D-0550) as well as all other chemicals were supplied by Sigma -Aldrich (Taufkirchen, Germany). Carbon dioxide 4.5 (purity 99,995 vol. %) was supplied by Messer MG Ruše, Slovenia.

Methods:

Analytical method

For determination of glucose content in the reaction mixture, UV spectrophotometric method was used [4].
This assay involves the measurement of reducing sugars, estimated as glucose equivalents, by the dinitrosalicylic acid (DNS) method.

Immobilization

Immobilization process of the enzymes into the matrix of silica aerogels includes two steps [5]. The detailed method of entrapment of enzymes by sol–gel synthesis was already described in our previous paper [6].

Gels were dried by two different drying routes:

Conventional drying (xerogels)

Alcohol was evaporated from the sol-gel matrix by drying in the air at 40 °C.

Supercritical drying with CO_2 (aerogels)

In supercritical carbon dioxide drying (Figure 1), alcohol was replaced by carbon dioxide. Alcohol was extracted with carbon dioxide above the critical conditions of CO_2. Experimental conditions during supercritical drying with carbon dioxide were: T=40 °C and p=100 bar.
During SC drying with CO_2 only one (liquid or supercritical) phase was present. At these conditions the capillary forces disappear and the structure of aerogels was under very little internal stresses. At the end of the extraction only CO_2 remains and during the depressurization step ($\Delta P/\Delta t$ = 3 bar/min) CO_2 has to pass from supercritical phase directly into gas phase.

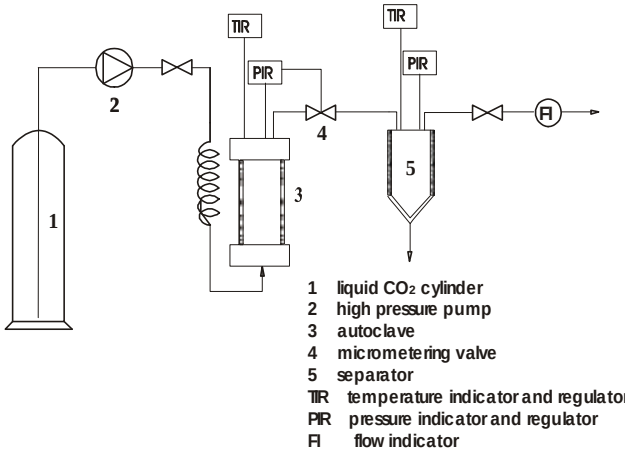

Figure 1: Experimental apparatus for SC CO_2 drying.

Determination of immobilized cellulase thermal stability

Crude enzyme preparation, aerogel and xerogel enzyme preparations were incubated at different temperatures for 24 hours in an autoclave in SC CO_2. After slow depressurization residual enzyme activities were determined.

II – RESULTS

Comparison of thermal stabilities of on sol-gel matrix immobilized cellulase

A certain amount of immobilized enzyme preparation was exposed to SC CO_2 at different temperatures for 24 hours. Cellulase in its free form was also parallel exposed to SC CO_2. Enzyme preparation, recovered from the reactor after slow depressurization, was used as catalyst for hydrolysis of carboxymethyl-cellulose (CMC) in non-solvent system in the batch reactor at 30°C and atmospheric pressure. For the comparison the same hydrolysis reaction was also catalyzed by the non-incubated free cellulase, as well as non-immobilized aerogel and xerogel enzyme preparations. Conversion was determined after 1,5 hours. Enzyme stability was determined as a difference between conversion of the reaction, catalyzed by fresh cellulase (not exposed to high-pressure) and that of reaction, catalyzed by immobilized cellulase, treated with carbon dioxide.

From Figure 2 it can be clearly seen that at atmospheric pressure activities of both immobilized enzyme preparations are higher then the activity of the crude enzyme preparation. In the case of in SC CO_2 preincubated aerogel enzyme preparation its activity significantly increased. Activity of the in SC CO_2 (at 100 bar and 300 bar) preincubated aerogel enzyme preparation was much higher then the activity of crude enzyme preparation at atmospheric pressure and also at different temperatures in SC CO_2 preincubated crude enzyme preparation (Figure 2). As expected, activity of the preincubated aerogel enzyme preparation (at 100 bar and 300 bar) and preincubated crude enzyme preparation decreased with the temperature increase from 35ºC to 110ºC. However, activity of the preincubated aerogel enzyme preparation at 110 °C was still higher then the residual activity of crude enzyme preparation at atmospheric pressure.

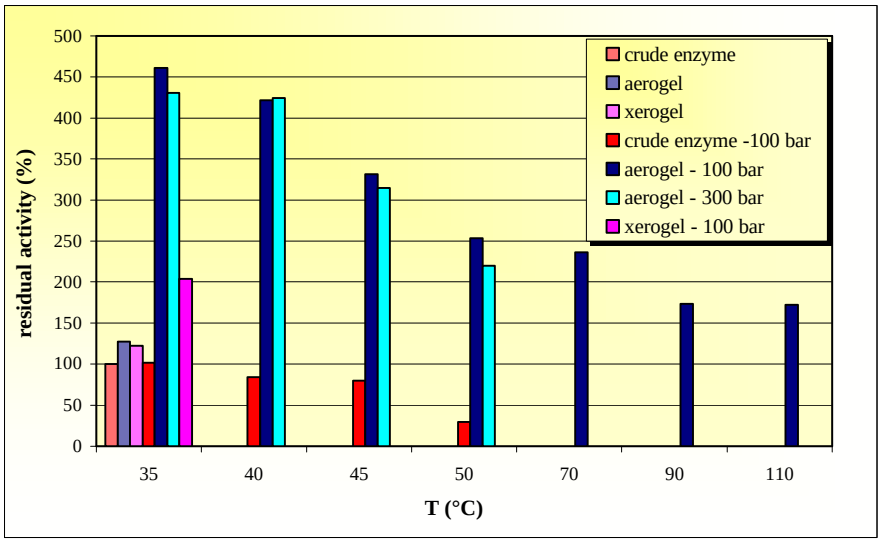

Figure 2: Residual activity of the immobilized cellulase after 24 h incubation at different temperatures in SC CO_2 at 100 bar and 300 bar. Comparison of thermal stabilities of aerogel, xerogel and crude enzyme preparations. Lined columns represent activity of crude enzyme and residual activities of aerogel and xerogel enzyme preparations at atmospheric pressure without preincubation in SC CO_2. Conversion was determined after 1,5 hours.

Influence of the reuse of immobilized enzyme on the activity of the cellulase

When immobilization techniques are studied, the knowledge about the stability of immobilized enzyme preparation is very important. Therefore the same enzyme preparation (aerogel or xerogel preparation) was used as biocatalyst for many cycles.

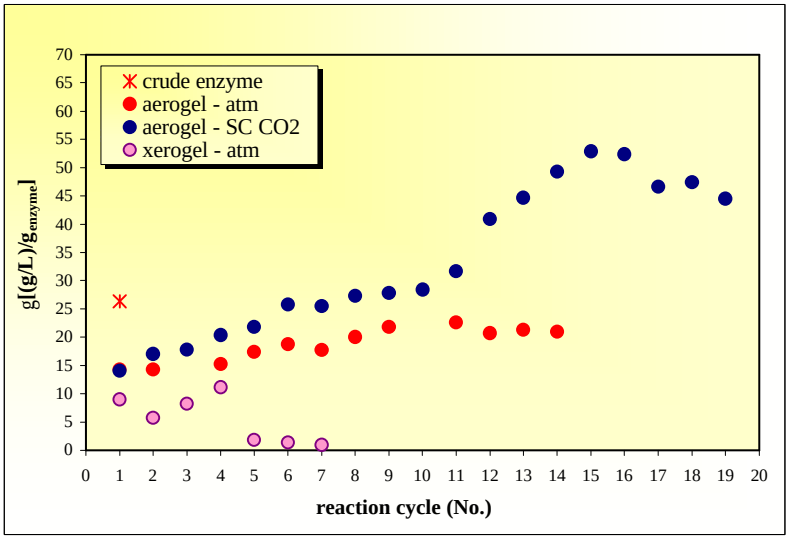

Figure 3: Stabilities of aerogel and xerogel enzyme preparations at atmospheric pressure and in SC CO_2. One cycle lasted for 5 hours. Symbols: atm - reaction was performed at atmospheric pressure, SC CO_2 - reaction was performed in SC CO_2.

One cycle means one reaction, performed in the batch stirred tank reactor, which lasted for 5 hours. After each reaction, immobilized enzyme preparation was washed three times with water. Figure 3 shows changes in concentrations of glucose at the hydrolysis of CMC with the number of reaction cycles.
Reactions were performed at atmospheric pressure at 35°C and in SC CO_2 at 35°C and 100 bar. Concentration of glucose at the reaction catalyzed by on aerogel immobilized-enzyme in SC CO_2 was lower then the concentration of glucose at the reaction catalyzed by crude enzyme preparation till 6^{th} reaction cycle. After this cycle, the concentration of product increased with number of reuse of aerogel enzyme preparation till 15^{th} reaction cycle, when the decrease in concentration of glucose was observed.
When the reaction was carried out at atmospheric pressure, concentration of glucose at reaction catalyzed by aerogel enzyme preparation was during all reaction cycles lower then the concentration of glucose at reaction, catalyzed by crude enzyme preparation.

Figure 4 shows the influence of the immobilization and reaction medium on the concentration of glucose (product) at 14^{th} cycle of aerogel enzyme preparation reuse.
Reactions were performed at 35°C and atmospheric pressure and at 100 bar and 35°C in CO_2. They were followed for 6 hours.
Initial reaction rate of in SC CO_2 performed reaction was lower then initial reaction rate of reaction performed at atmospheric pressure. However, final concentration of the product was 2-times higher in the case of using SC CO_2 as reaction medium. Reaction catalyzed by aerogel enzyme peraparation at atmospheric pressure has similar course as reaction catalyzed by crude enzyme.

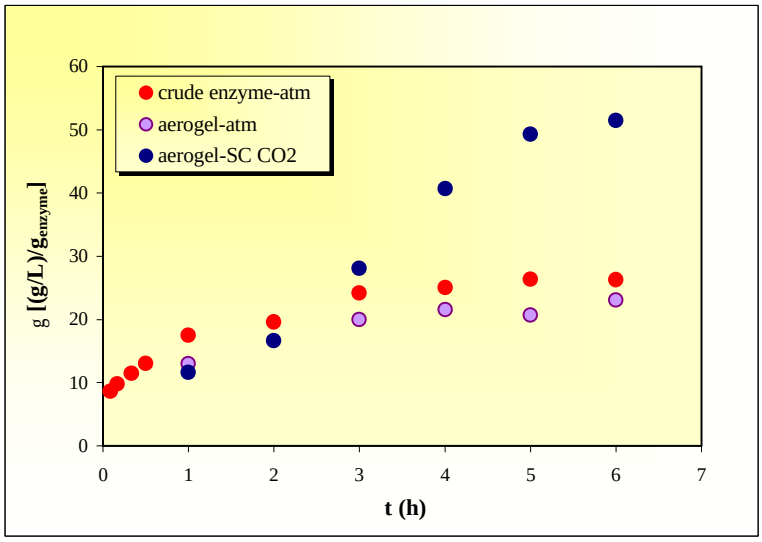

Figure 4: Influence of immobilization and reaction medium on the concentration of glucose. Hydrolysis of CMC was catalyzed by crude cellulase preparation and cellulase preparation immobilized on SiO_2 carrier. The reaction was performed at 35°C and atmospheric pressure and at 100 bar and 35°C in CO_2.

CONCLUSION

The immobilization technique of cellulase into silica aerogel matrix has proved very applicable especially when SC CO_2 was used as reaction medium. The reason for very high relative enzyme activities in the case of SCF as a reaction medium was the high dispersion of the cellulase in the silica aerogel matrix.
The investigation have shown, that aerogel enzyme preparations were superior to xerogel preparation and were successfully reused for at least 20 times in the high-pressure batch system.

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