MATEJA PRIMOŽIČ
MAJA HABULIN
MUZAFERA PALJEVAC
ŽELJKO KNEZ

University of Maribor, Faculty of Chemistry and Chemical Engineering, Laboratory for Separation Processes, Maribor, Slovenia

SCIENTIFIC PAPER

612.015.1+66.097:66-987:66.023

# ENZYME–CATALYZED REACTIONS IN DIFFERENT TYPES OF HIGH–PRESSURE ENZYMATIC REACTORS

*The enzyme–catalyzed hydrolysis of carboxy–methyl cellulose (CMC) was performed in three different types of reactors; in a batch stirred–tank reactor (BSTR) operating at atmospheric pressure, in a high–pressure batch stirred–tank reactor (HP BSTR) and in a high–pressure continuous tubular–membrane reactor (HP CTMR). In the high–pressure reactors aqueous SC $CO_2$ was used as the reaction medium. The aim of our research was optimization of the reaction parameters for reaction performance. All the reactions were catalyzed by cellulase from Humicola insolens. Glucose production in the high–pressure batch stirred–tank reactor was faster than in the BSTR at atmospheric pressure. The optimal temperature for the reaction performed in the BSTR at atmospheric pressure was 30 $^{o}$C, while the optimal temperature for the reaction performed in SC $CO_2$ was 32 $^{o}$C. The influence of the application of tubular ceramic membranes in the high–pressure reaction system was studied on the model reaction of CMC hydrolysis at atmospheric pressure and in SC $CO_2$. The reaction was catalyzed by cellulase from Humicola insolens covalently linked to the surface of the ceramic membrane. The hydrolysis of CMC in SC $CO_2$ and at atmospheric pressure was performed for a long time period. The reaction carried out in SC $CO_2$ was more productive than the reaction performed at atmospheric pressure.*

*Key words: Enzymatic reaction, Cellulase, high–pressure, Supercritical carbon dioxide, high–pressure batch reactor, High–pressure membrane reactor.*

Cellulose is a beta–linked glucose polymer, whereas hemicellulose is a highly branched chain of xylose and arabinose that also contains glucose, mannose and galactose [1]. Especially the use of enzymatic hydrolysis to convert cellulose and hemicellulose into fermentable sugars has been studied extensively, as this area has a great potential for improving the economical feasibility of bioethanol production [2].

The conversion of lignocellulosic material into bioethanol has gained increasing attention in recent years due to growing concerns about the shortage of fossil fuel and interest in the domestic production of fuel [3].

In general, the hydrolysis of cellulose is more difficult to achieve than that of other polysaccharides. The difficulties involve a slow reaction rate, the lack of an ideal reactor system, the complexity of interfacial heterogeneous hydrolysis influenced by the structure and composition of cellulosic materials, cellulase adsorption and desorption, enzyme inhibition by cellobiose and glucose etc [4].

The realization that most enzymes can function perfectly well under nearly anhydrous conditions and, additionally, display a number of useful properties, e.g., highly enhanced stability and different selectivity, has dramatically widened the scope of their application in organic synthesis. The use of monophasic organic solvents can be problematic because of toxicity, flammability and increasing environmental concerns. Therefore, supercritical fluids (SCFs) have attracted much attention in recent years as an alternative to organic solvents for carrying out enzymatic reactions. The use of SCFs as solvents for enzymatic transformations is a relatively new area of research, which is expected to expand in the future.

SCFs, such as carbon dioxide (SC $CO_2$), exhibit properties similar to organic solvents, but with the additional capacity of encouraging transport phenomena and facilitating reaction product separation by tuning the solvent power [5]. The use of SCFs decreases the mass transfer limitations because of the high diffusivity of reactants in the SC medium, the low surface tension, and because of the relatively low viscosity of the mixture.

SC $CO_2$ has been most frequently used as a supercritical medium for biotrasformations: its critical pressure (73.8 bar) and its critical temperature (31.1$^{o}$C) are consistent with the use of enzymes [6]. The high diffusivity of SCF and low surface tension lead to reduced internal mass transfer limitations for heterogeneous chemical or biochemical catalysis.

The physico–chemical properties of dense gases are determined by their pressure and temperature, and are especially sensitive near their critical point. By reducing the solvent–power of a dense gas in several stages, the fractionation of the product and unreacted reactants is possible.

Paper presented at the 1$^{st}$ South–East European Congress of Chemical Engineering, Belgrade, September 25–28, 2005

Author address: Ž. Knez, University of Maribor, Faculty of Chemistry and Chemical Engineering, Laboratory for Separation Processes, Smetanova 17, 2000 Maribor, Slovenia
E–mail: zeljko.knez@uni–mb.si
Paper received: October 31, 2005
Paper accepted: March 20, 2006

Most of the time enzymatic reactions are carried out in a conventional enzyme batch reactor at controlled temperature. These types of reactors are suitable for the preliminary screening of enzyme reactions in SCFs. They are cheaper and much more easily controlled than various flow reactor types [6]. Continuous processes are very interesting especially for industry, because they are more cost efficient and the reactors can be kept smaller in size. The usage of membrane bioreactors is especially beneficial for polymer degradation processes, where the low molar mass product may have an inhibitory effect, but can be separated easily by a proper porous membrane. This is the case of the enzymatic hydrolysis of polysaccharides, such as cellulose or starch. The long polysaccharide chain, as well as the biocatalyst (enzyme or cell), are rejected by the membrane, while the product (glucose) passes through the membrane into the other phase. In such a system, the continuous uptake of substrate and the release of product without loss of biocatalysts can be achieved [7].

When the enzyme is pressurized in the supercritical fluid it enters the enzyme by diffusion. Usually the diffusion is relatively slow and after a certain time the enzyme is saturated with the fluid. The expansion may cause a pressure difference between the enzyme and the system. Such disadvantages could be overcome by using continuous reactors.

Some results of our research on the cellulase–catalyzed hydrolysis of CMC are presented in this paper. The influence of temperature on the reaction at atmospheric pressure and the influence of various temperature – pressure combinations on the reaction in SC $CO_2$ were investigated. The enzyme–catalyzed hydrolysis of CMC was also performed in a high–pressure continuous tubular membrane reactor (HP CTMR), where the membrane was used as a separation unit to retain the biocatalyst in the system.

## MATERIALS AND METHODS

Cellulase from *Humicola insolens* (Fluka 22175, 70 U/g) was supplied by Sigma–Aldrich, Germany and cellulase – Celluzyme 0.7T – was kindly donated by NOVO Nordisk A/S, Bagsvaerd. Carboxy–methyl cellulose (C–4146), 3,5 dinitrosalycic acid (D–0550), as well as all the other chemicals were supplied by Sigma–Aldrich, Germany. Ceramic membranes for the reactions in HP CTMR were supplied by TAMI Industries, Germany. Carbon dioxide 4.5 (purity 99.995 vol.%) was supplied by Messer MG Ruše, Slovenia.

### Assay procedure for glucose determination

The assay is based on the measurement of reducing sugars, estimated as glucose equivalents, by the dinitrosalicylic acid (DNS) method.

1 mL of reaction mixture (taken at defined times) was pipetted into a tube (sample) and immediately 3 mL of DNS were added, to stop the reaction. As a blank sample, 3 mL of DNS and 1 mL of reaction mixture, which was of the same composition as at time zero, were pipetted into the tube and mixed. As a glucose standard, 3 mL of DNS and 1 mL of glucose standard were mixed.

All the tubes were placed in a boiling water bath for exactly 5 minutes and then in a cold–water bath for 5 minutes.

The absorbance (OD) of all the samples against a colour blank (3 mL of DNS and 1 mL of water), using a spectrophotometer set at 540 nm, was determined.

The glucose concentration was calculated from the following equation:

$$\text{Glucose concentration} = \frac{(OD_{sample} - OD_{blank\,sample}) \cdot 0.5}{OD_{glucose\ standard}}, \text{ mg/mL}$$

### Enzyme–catalyzed hydrolysis of CMC performed in a high–pressure batch stirred–tank reactor (HP BSTR) at atmospheric pressure and in SC $CO_2$

Batch stirred–tank reactors are usually used for screening enzymatic reactions. The simple scheme of the high–pressure system is shown in Figure 1.

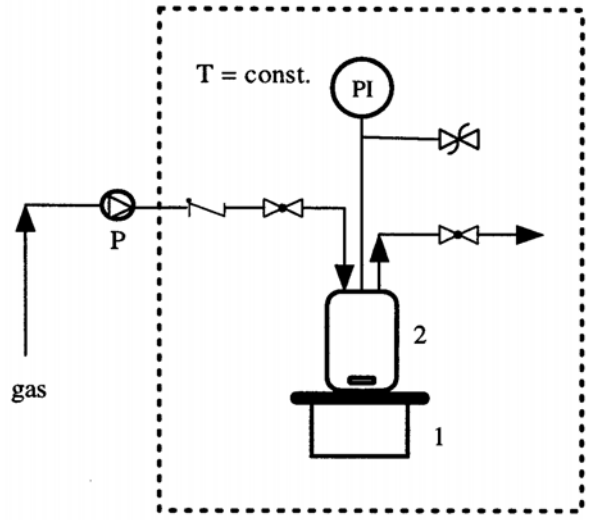


*Figure 1. The experimental batch–stirred–tank apparatus for synthesis under high pressure. 1 – magnetic stirrer and heater; 2 – reactor; P – high pressure pump; PI – pressure indicator*

The volume of the reactor, which was designed for operation up to 500 bar, was 80 mL. The reaction mixture (CMC solution) was stirred and heated up to the operational temperature in an oil bath. When the CMC solution in the reactor was termostated to the required temperature, a certain concentration of cellulase preparation was added. Dry $CO_2$ was then, pumped into the reactor up to the desired pressure in the case when the reaction was performed in the dense gas. During the

reaction samples were taken from the reactor and the glucose concentration was determined.

### Enzyme–catalyzed hydrolysis of CMC performed in a high–pressure continuous tubular–membrane reactor (HP CTMR) at atmospheric pressure and in SC $CO_2$

1 g of cellulase from *Humicola insolens* (Cellulase from Humicola insolens, Fluka 22175, Switzerland; 70 U/g) was covalently bound [8] on a ceramic membrane INSIDE CéRAM (TAMI–Industries GmbH, Germany). After the immobilization procedure, the enzyme–catalyzed hydrolysis of CMC was performed in a high–pressure continuous enzymatic tubular membrane reactor [9].

At first, the reaction was performed at atmospheric pressure and the following reaction parameters: the CMC concentration was 15 g/L, the operating temperature 45 °C and the flow rate of the substrate 0.5 mL/min. The reaction was continuously performed for a long time period.

The same reaction was also performed in SC $CO_2$ at 100 bar and 45 °C. The other reaction parameters were the same as in the first experiment. The flow rate of $CO_2$ was 2.5 L/h. During the reaction samples were taken from the reactor in a sampling tube and the glucose concentration was determined by the previously described method.

## RESULTS AND DISCUSSION

### The influence of temperature on the enzyme–catalyzed hydrolysis of CMC at atmospheric pressure, performed in a BSTR and in SC $CO_2$ at different pressures, performed in a HP BSTR

The temperature, as one of the most important reaction parameters, was optimized. The rate of enzyme catalyzed reactions increases with increasing temperature. This effect was also observed in the case of the cellulase–catalyzed hydrolysis of CMC at atmospheric pressure and in SC $CO_2$. The reactions were performed in the temperature range between 20 °C and 70 °C, when the reaction was performed at atmospheric pressure and in the range between 32 °C and 60 °C, when the reaction was carried out in SC $CO_2$ at 200 bar.

Thermal activation between 20°C and 30°C was observed for the reaction performed at atmospheric pressure. In the case when the temperature was further increased, enzyme deactivation occurred (Figure 2). When the reaction was performed at 70°C, was still some cellulase activity. On the basis of the slope of the straight lines in the Arrhenius diagram, an activation energy ($E_a$) of 42.36 kJ/mol was calculated [10].

When supercritical fluids are used as the reaction medium, the optimal temperature also depends on the operational pressure. With changes in pressure and

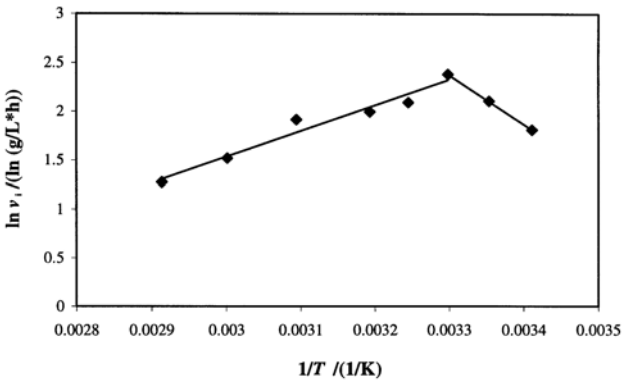


*Figure 2. Arrhenius plot for the hydrolysis of CMC at atmospheric pressure, catalyzed by cellulase from Humicola insolens. Reactions at different temperatures were performed in a BSTR. Reaction parameters: γ(enzyme) = 44 g/L, γ(CMC) = 15 g/L, pH = 4.6, n = 600 min$^{-1}$*

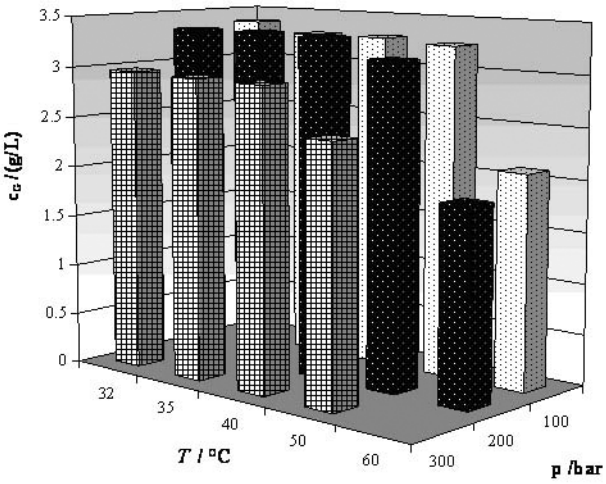


*Figure 3. The influence of temperature and pressure on glucose production after 5 hours of reaction, when the hydrolysis of CMC was carried out in SC $CO_2$. The reactions were performed in HP BSTR. Reaction parameters: γ(enzyme) = 44 g/L, γ(CMC) = 15 g/L, pH = 4.6, n = 600 min$^{-1}$*

temperature the transport properties of SCF may significantly change, which results in changes in the reaction rates.

Experiments at different temperature and pressure combinations in SC $CO_2$ showed that the highest activity of Celluzyme 0.7T after 5 hours of reaction was obtained at 32 °C and 200 bar (Figure 3).

No significant differences in glucose production at the same temperature were observed when the reaction was performed at 200 bar or at 100 bar. At 300 bar the reaction was found to proceed more slowly than at the other two pressures at all the chosen temperatures. By changing the pressure, one simultaneously changes $CO_2$ the density. The reaction rate may change with the $CO_2$ density because the physical parameters, such as the dielectric constant, change with density. These changes may indirectly influence the enzyme activity.

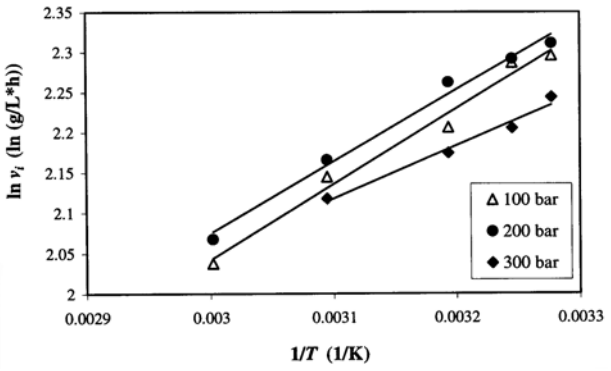


*Figure 4. Arrhenius plot for the hydrolysis of CMC in SC $CO_2$ at different pressures, catalyzed by cellulase from Humicola insolens. Reactions at different temperatures were performed in a HP BSTR. Reaction parameters: γ(enzyme) = 44 g/L, γ(CMC) = 15 g/L, pH = 4.6, n = 600 $min^{-1}$*

It may be seen from the Arrhenius plot for the enzyme–catalyzed hydrolysis of CMC in SC $CO_2$ (Figure 4) that enzyme deactivation occurred in the temperature range between 32 °C and 60 °C (apart from the chosen pressure).

### Comparison of the cellulase–catalyzed hydrolysis of CMC at atmospheric pressure and in SC $CO_2$ under optimal conditions performed in a HP BSTR

Figure 5 shows the product concentration of the enzyme–catalyzed hydrolysis of CMC at atmospheric pressure and for the same reaction performed in SC $CO_2$. A higher reaction rate was obtained in SC $CO_2$. At atmospheric pressure the highest glucose concentration in the reaction mixture after 3 h was around 2.5 mg/mL, while in SC $CO_2$ after the same time it was around 3 mg/mL. The difference may have occurred due to lower mass transfer limitations achieved in SC $CO_2$. The external mass transfer limitation was reduced to a minimum in SC $CO_2$.

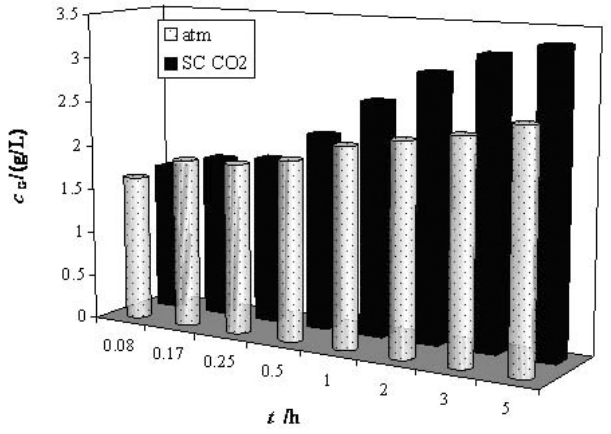


*Figure 5. Glucose production under optimal conditions as a function of time at atmospheric pressure and in SC $CO_2$. The reactions were performed in HP BSTR. Reaction parameters: at atmospheric pressure: γ(enzyme) = 44 g/L, γ(CMC) = 15 g/L, pH = 4.6, n = 600 $min^{-1}$, T = 30 °C; in SC $CO_2$: γ(enzyme) = 44 g/L, γ(CMC) = 15 g/L, pH = 4.6, n = 600 $min^{-1}$, T = 32 °C, p = 200 bar.*

### Reactions in a high–pressure continuous tubular membrane reactor (HP CTMR)

Tubular ceramic membranes were used, as a separation unit in the membrane reactor.

The application of tubular ceramic membranes in a high–pressure reaction system was studied on the hydrolysis of carboxy–methyl cellulose (CMC) at atmospheric pressure and in SC $CO_2$, catalyzed by cellulase from *Humicola insolens* with covalently linked on the surface of the ceramic membrane [9].

The reaction carried out in SC $CO_2$ gave higher productivity than the reaction performed at atmospheric pressure (Figure 6). The hydrolysis of CMC in SC $CO_2$ and atmospheric pressure was performed for a long time period. In both cases the concentration of product slowly decreased with time.

At first the enzyme–catalyzed hydrolysis of CMC in a tubular enzymatic membrane reactor was performed at atmospheric pressure at 45 °C. The concentration of reduced glucose slowly decreased during the reaction, but some productivity was still perceived after 42 hours of reaction.

The same reaction was also performed at 45 °C and 100 bar in SC $CO_2$. The transmembrane pressure was low (around 2 bar), due to the possibility of the breakdown of the membrane structure.

The concentration of reduced sugar was constant for the first 9 hours of the reaction and was around 12 mg/L. A slow decrease in the concentration of the product was observed after that. The concentration of product after 46 hours of reaction in SC $CO_2$ was still higher than the concentration of reduced glucose achieved in the reaction performed at atmospheric pressure. The reason for the decrease in the

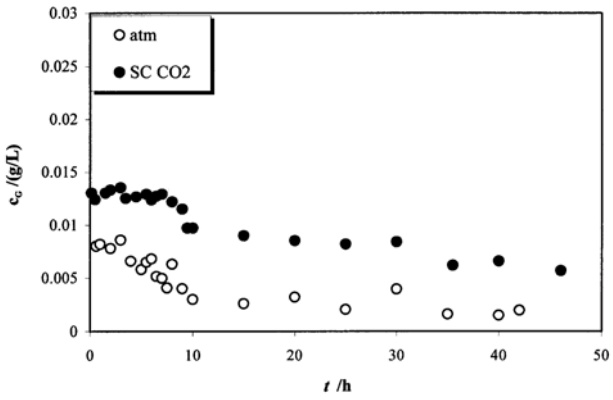


*Figure 6. Enzyme catalyzed hydrolysis of CMC at atmospheric pressure and in SC $CO_2$. The reactions were performed in a high–pressure continuous tubular membrane reactor. Reaction parameters: at atmospheric pressure; T = 45 °C, flow rate of the substrate 0.5 mL/min; in SC $CO_2$; T = 45 °C, p = 100 bar, flow rate of the substrate 0.5 mL/min and flow rate of the SC $CO_2$ 2.5 L/h*

concentration of the formed product during the reaction performed for a long time was most likely due to the damage of the enzyme active centre during the immobilization processes.

The high diffusivity of SCFs and their low surface tension led to reduced internal mass–transfer limitations and, therefore, an increase in the reaction rate of the reaction performed in SC $CO_2$ could appear. The ceramic membranes showed good stability in SC $CO_2$ and served very well as a support for the biocatalyst and as a separation unit.

## CONCLUSION

It was established that cellulase from *Humicola insolens* is very sensitive to temperature, because the optimal temperature for the reaction at atmospheric pressure was 30 °C. At higher temperatures enzyme deactivation occurred. The activation energy was calculated from the Arrehnius plot 42.36 kJ/mol.

The enzyme–catalyzed hydrolysis of CMC was also performed in SC $CO_2$, where, despite the bad solubility of CMC in SC $CO_2$, higher reaction rates were observed. The reason for the improvement in the reaction rate are the transport properties of SC $CO_2$, resulting in lower mass–transfer limitations.

The application of tubular ceramic membranes in the high–pressure reaction system was studied on the hydrolysis of carboxy–methyl cellulose (CMC) at atmospheric pressure and in SC $CO_2$, catalyzed with cellulase covalently linked on the surface of the ceramic membrane. The reaction carried out in SC $CO_2$ gave higher productivity than the reaction performed at atmospheric pressure.

## REFERENCES


[1] A. Weislogel, S. Tyson, D. Johnson, Handbook on Bioethanol: Production and Utilization, C. Wyman (Ed.), Taylor and Francis, Washington DC 1996 pp. 105–118

[2] H. JØrgensen, L. Olsson, Production of cellulases by Penicillium brasilianum IBT 20888 ( Effect of substrate on hydrolytic performance, Enzyme Microb. Technol., In Press, Available online 15 August 2005

[3] J. Sheehan, M.E. Himmel, Enzymes, energy, and the environment: a strategic perspective on the U.S. Department of Energy's research and development activities for ethanol, Biotechnol. Prog. **15** (1999) 817–827.

[4] E.O. Jones, M.J. Lee, Kinetic analysis of bioconversion of cellulose in an attrition bioreactor, Biotechnol. Bioeng. **31** (1988) 35–43

[5] P. Lozano, T. de Diego, D. Carrie, M. Vaultier, J.L. Iborra, Continuous green biocatalytic processes using ionic liquids and supercritical carbon dioxide, Chem. Commun. **7** (2002) 692–693

[6] O. Aaltonen, Enzymatic Catalysis in Chemical Synthesis using Supecritical Fluids, P.G. Jessop, and W. Leiner, (Eds.), Wiley–VCH, Weinheim 1999, p. 415

[7] K. Bélafi–Bakóa, A. Koutinasb, N. Nemestóthya, L. Gubiczaa, C. Webbb, Continuous enzymatic cellulose hydrolysis in a tubular membrane bioreactor, Enzyme Microb. Technol., In Press, Available online 1 August 2005

[8] P. Lozano, A.B. Perez–Marin, T. De Diego, D. Gomez, D. Paolucci–Jeanjean, M.P. Belleville, G.M. Rios, J.L. Iborra, Active membranes coated with immobilized Candida antarctica lipase B: preparation and application for continuous butyl butyrate synthesis in organic media, J. Membrane Sci. **201** (2002) 55–64

[9] M. Habulin, M. Primožič, Ž. Knez, Enzymatic reactions in high–pressure membrane reactors, Ind. Eng. Chem. Research, In Press, Accepted 17.06.2005

[10] A. Cornish–Bowden, Fundamentals of Enzyme Kinetics, Portland Press, London 1995


## IZVOD

ANALIZA ENZIMSKI KATALIZOVANIH REAKCIJA U RAZLIČITIM TIPOVIMA REAKTORA KOJI RADE POD VISOKIM PRITISCIMA

(Naučni rad)

Mateja Primožič, Maja Habulin, Muzafera Paljevac, Željko Knez
Univerzitet u Mariboru, Fakultet za hemiju i hemijsko inženjerstvo, Laboratorija za separacione procese, Maribor, Slovenija

Ispitivana je enzimski katalizovana hidroliza karboksimetil celuloze (CMC) u tri različita tipa reaktora: šaržnom reaktoru pod atmosferskim pritiskom (BSTR) I visokim pritiskom (HP BSTR) kao i u cevnom membranskom reaktoru pod visokim pritiskom (HP CTMR). U reaktorima koji su radili pod visokim pritiscima korišćena je voda i natkritični ugljen dioksid (SC $CO_2$) kao reakcioni medijum. Cilj ovih ispitivanja bio je da se optimizuju reakcioni uslovi za ovu reakciju. Svi eksperimenti su realizovani korišćenjem enzima celulaze *Humicola insolens*. Dobijanje glukoze u šaržnom reaktoru pod visokim pritiskom brže je nego u istom tipu reaktora pod atmosferskim pritiskom. Optimalna temperatura za proces pod atmosferskim pritiskom je 30 °C dok je u slučaju prisustva SC $CO_2$ 32 °C. Primena cevnog reaktora sa keramičkim membranama pod visokim pritiskom analizirana je korišćenjem model reakcije hidrolize CMC pod atmosferskim pritiskom i pod visokim pritiskom u prisustvu SC $CO_2$. Reakcija je katalizovana površinom keramičke membrane na koju je kovalentnim vezama imobilisana lipaza izolovana iz *Humicola insolens*. Hidroliza CMC u prisustvu SC $CO_2$ i pod atmosferskim pritiskom praćena je u dužem vremenskom procesu pod stacionarnim uslovima. Veća produktivnost ostvarena je tokom reakcije realizovane u prisustvu SC $CO_2$ nego kada je reakcija sprovedena pod atmosferskim pritiskom.

Ključne reči: Enzimska reakcija, Hidroliza, Karboksimetil celuloza, Celulaza, Visoki pritisci, Natkritični uslovi, Ugljen dioksid, Šaržni reaktor, Membranski cevni reaktor, Imobilisan enzim.