CHEMICAL ENGINEERING TRANSACTIONS
VOL. 27, 2012
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at: www.aidic.it/cet
Guest Editors: Enrico Bardone, Alberto Brucato, Tajalli Keshavarz

ISBN 978-88-95608-18-1; **ISSN** 1974-9791
DOI: 10.3303/CET1227040

Immobilization of Cellulase for Industrial Production

Gordana Hojnik Podrepšek, Mateja Primožič, Željko Knez, Maja Habulin*

University of Maribor, Faculty of Chemistry and Chemical Engineering, Laboratory for Separation Processes and Product Design, Smetanova ul. 17, 2000 Maribor, Slovenia
maja.habulin@uni-mb.si

Immobilized enzymes are used in analytical chemistry and as catalysts for the production of chemicals, pharmaceuticals and food. Because of their particular structure, immobilized enzymes require optimal conditions, different from those of soluble enzymes. Particle size, particle-size distribution, mechanical and chemical structure, stability and the catalytic activity, used for immobilization, must be considered.
Generally, cellulases are used in various industries, including food, brewery and wine, agriculture, textile, detergent, animal feed, pulp and paper, and in research development. For the industrial application of cellulase, its immobilization, which allows the conditions of repeated use of the enzyme alongside retaining its activity, has been recently investigated. Celullase was immobilized with the use of glutaraldehyde, a covalent cross-linking agent in to cross-linked enzyme aggregates (CLEAs). The stability and activity of cross-linked cellulase, exposed to carbon dioxide under high pressure, were studied. Efficiency of enzyme immobilization was determined using Bradford method (Bradford, 1976). The activity of cross-linked cellulase was determined by spectrophotometric method.

1. Introduction

Cellulase, a multicomponent enzyme, consisting of three different enzymes (endocellulase, cellobiohydrolase and ß-glucosidase) is responsible for bioconversion of cellulose into soluble sugar (Zhou et al., 2009). Generally, cellulases are used in various applications, including food, brewery and wine, agriculture, textile, detergent, animal feed, pulp and paper industry, as well as in research development.
The technique of protein cross-linking by the reaction of glutaraldehyde with reactive NH_2 groups on the protein surface was initially developed in the 1960s (Sheldon, 2005). Glutaraldehyde is generally the cross-linking agent of choice as it is inexpensive and readily available in commercial quantities. Cross-linked enzyme aggregates (CLEAs) are selected to be a prominent route of enzyme immobilization technique, without the necessity of a solid support (Sheldon, 2005). Moreover, the cross-linked cellulase aggregates represent a suitable form of immobilized enzyme to be used in large-scale production processes and biotransformations, even on industrial scale. The cross-linked cellulase aggregates is carrier-free immobilized enzyme, in virtually pure cellulase and the negative effects of carriers can thus be avoided (Hanefeld, 2009).
In general practice, the procedure to prepare CLEAs includes two major steps that involve precipitation of soluble enzyme with suitable precipitant and crosslinking with an appropriate cross-linker, during which the particle size increases (Figure 1).

Please cite this article as: Hojnik Podrepsek G., Primozic M., Knez Z. and Habulin M., 2012, Immobilization of cellulase for industrial production, Chemical Engineering Transactions, 27, 235-240 DOI: 10.3303/CET1227040

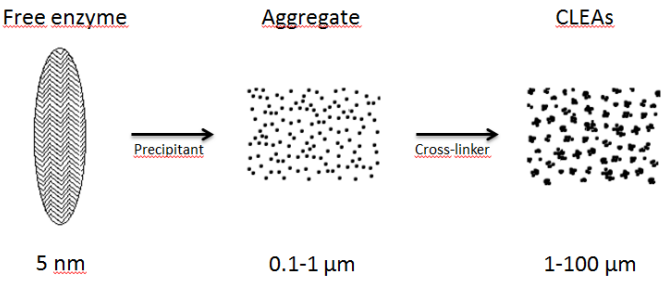

Figure 1: Preparation of CLEAs.

However, an ideal industrial immobilized enzyme has to meet several criteria, such as being recyclable, broadly applicable, cost effective and safe for use.
Supercritical carbon dioxide (SC CO_2) is by far the most studied supercritical fluids (SCF), because of its economical, technical, environmental, and health advantages. Moreover, it is an excellent solvent for the transport of hydrophobic compounds. Furthermore, its critical pressure is relatively low (7.34 MPa) and it has an ambient critical temperature (31 °C), which provides the mild conditions necessary for keeping its enzyme activity (Habulin et al., 2009). The effect of SC CO_2 on CLEAs activity at different temperatures and pressures was examined in our study.

2. Materials and methods

2.1 Materials

Cellulase (Cellusoft conc. L) (EC 3.2.1.4) was kindly donated from Novozymes A/S (Denmark). Egg albumin and pentaethylenehexamine (PEHA) were obtained from Acros Organics (Germany). Hydrogen peroxide solution (30 %) and acetone were purchased from Merck Chemical Company (Germany). Other reagents, including glutaraldehyde (25 %) and sodium cyanoborohydride solution ($NaBH_3CN$), were obtained from Sigma-Aldrich (Germany). All solutions were prepared freshly every day with MilliQ water. All other reagents were of analytical grade and were obtained either from Sigma-Aldrich or Acros Organics.

2.2 Methods

CLEA preparation

In general, the procedure to prepare CLEAs consisted of two steps: (1) physical aggregation/precipitation of the enzyme and (2) cross-linking. In this case, a cross-linking reaction followed by the addition of a reducing agent, sodium cyanoborohydride, was performed. All reactions were performed at room temperature (T = 25 °C) using pre-chilled solvents.
Enzyme precipitation was conducted using 90 % (v/v) of precipitation reagent and 10 % (v/v) of enzyme. Glutaraldehyde was used as a cross-linking agent. Cross-linking reaction was performed in the presence of PEHA and albumin. Cold $NaBH_3CN$ solution was used as the reducing agent. Finally, the CLEAs were recovered by centrifugation and were dispersed in 0.2 M phosphate buffer solution (PBS) and stored at 4 °C for subsequent activity analysis.
CLEAs were exposed at atmospheric pressure at 40 °C and 50 °C for desired treatment time (1, 2, 3, 4, 24, 48 h).

As a comparison, CLEAs were also exposed in a 120 mL high-pressure batch reactor at 40 °C and 50 °C to the desired pressure (10 MPa and 20 MPa). When the temperature in the high-pressure batch reactor with suspension of CLEAs reached 40 °C or 50 °C, the reactor was supplemented to the desired pressure with cooled CO_2. After the desired treatment time (1, 2, 3, 4, 24 h) the reactor was slowly depressured (0.3 MPa/min). The experiments were done in triplicate and the error bar represents the percentage error (±3%) in each set of readings.

3. Results and discussion

3.1 Particles size and shape of CLEAs

The number of enzyme molecules and the way they are packed together in an aggregate can be expected to have a crucial influence on the activity of the aggregate as a whole. A light microscope showed a very uniform structure of the aggregates (Figure 2), and the determined size of CLEAs was approximately 30-40 µm.

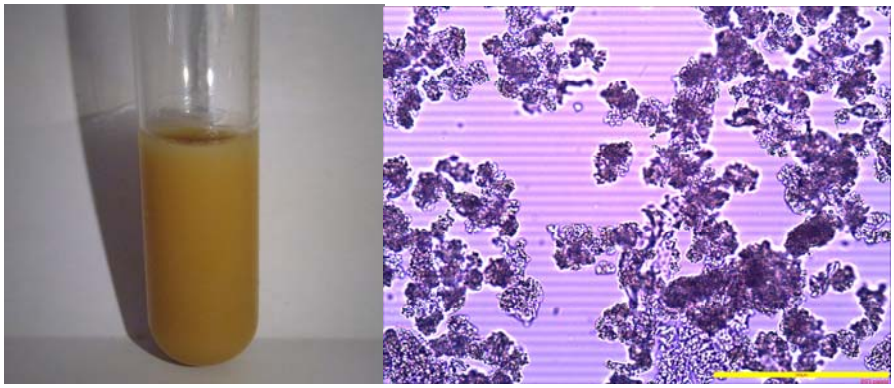

Figure 2: CLEA in tube and under a light microscope (magnification 400x).

3.2 Influence of temperature and treatment time on cellulase CLEAs activity under atmospheric pressure

Cellulase CLEAs were exposed at atmospheric pressure at 40 °C and 50 °C for defined time (Figure 3). Their activity decreased after 48 hours of exposure to 40 °C. However, CLEAs activity increased after 4 hours of exposure to 50 °C and then by lengthening the treatment time at atmospheric pressure it decreased.

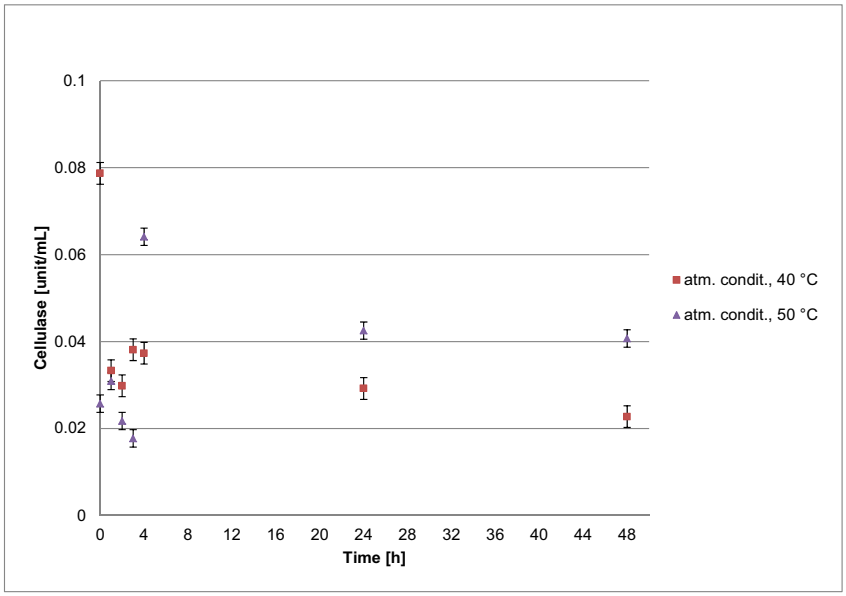

Figure 3: Cellulase activity after exposure under atmospheric pressure. Percentage error in each set of readings was ±3%.

3.3 Influence of pressure, temperature and treatment time during SC CO_2 treatment on cellulase CLEAs activity

Influence of depressurization rate on the activity of immobilized cellulase (CLEAs) exposed to compressed CO_2 was investigated (Figure 4 and 5). It was found out that the activity of CLEAs decreased with increasing pressure. The CLEAs activity after the exposure to SC CO_2 at 20 MPa and 40 °C was lower than at 10 MPa and 40°C, which indicated that higher pressure reduces the CLEAs activity (Figure 4).

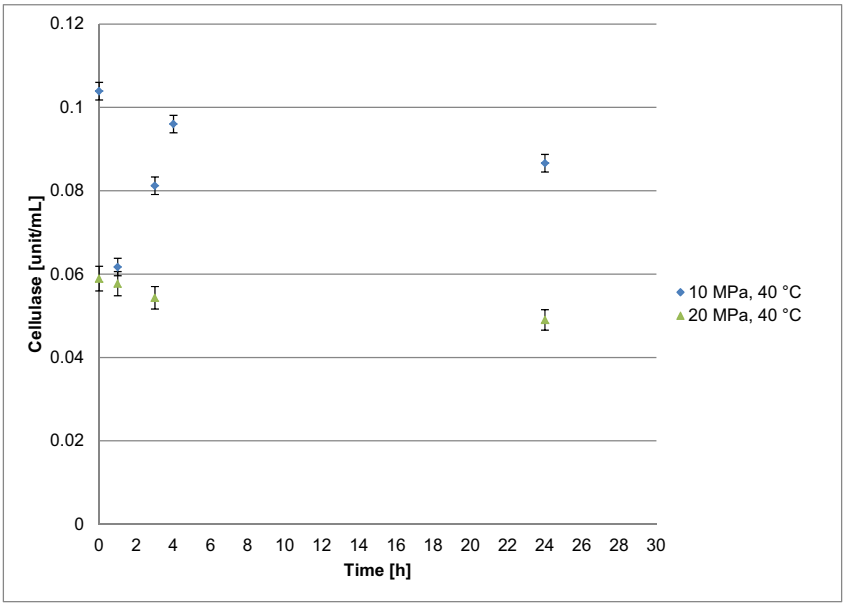

Figure 4: Cellulase activity after exposure in SC CO_2 at 40 °C. Percentage error in each set of readings was ±3%.

The highest value of CLEAs activity (0.1022 unit/mL) at 10 MPa and 50 °C was reached after 3 hours of exposure of the enzyme preparation at these conditions. By lengthening the treatment time the activity of CLEAs decreased (Figure 4).

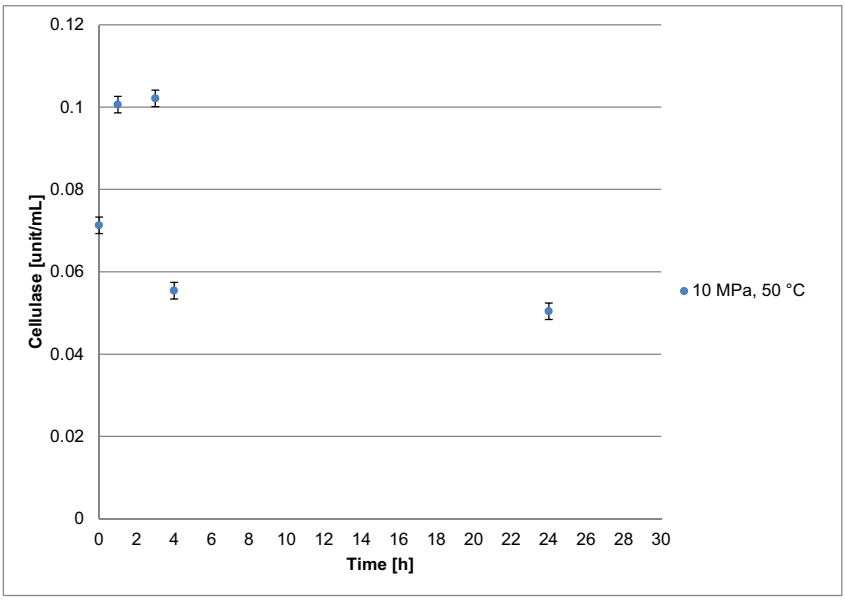

Figure 5: Cellulase activity after exposure in SC CO_2 at 50 °C. Percentage error in each set of readings was ±3%.

When the enzyme is used in a high-pressure batch reactor, pressurization/depressurization step influence its activity. If the expansion is too rapid, unfolding of the enzyme may occur and destroy the enzyme structure. Therefore, during the experiments the slow depressurization (0.3 MPa/min) was used.

Glutaraldehyde is generally the cross-linking agent of choice as it is inexpensive and readily available in commercial quantities (Sheldon et al., 2007). However, we determined lower activity of immobilized enzyme in the comparison with the activity of non-immobilized enzyme, which might be caused by the reaction of cross-linker with amino acid residues that are crucial for the activity of the enzyme.

4. Conclusions

Cellulase CLEAs were successfully prepared. The CLEA technology has many advantages in the context of industrial applications. Immobilization as cross-linked enzyme aggregates (CLEAs) is highly attractive due to its simplicity, robustness, wide applicability, operational stability, is hypoallergenic, has no necessity for highly pure enzyme, is easy recoverable and recyclable and has high activity and productivity. The spherical structure of CLEAs offers new insights in the CLEAs behavior and possess high surface area and hence more catalytic sites, which is advantageous as a biocatalyst. A major challenge in industrial biotransformations is the development of a stable, robust and preferably recyclable biocatalyst. The method is exquisitely simple and amenable to rapid optimization, which translates to low costs and short time-to-market. We believe that CLEAs will, in the future, be widely applied in industrial biotransformations and other areas requiring immobilized enzymes.

References

Bradford, M.M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analitical biochemistry, 72, 248-254.

Habulin M., Primožič M., Knez Ž., 2009, Modern biocatalysis, Kinetic resolution of sec-alcohol in non-conventional media. Wiley-VCH, Weinheim, Germany.

Habulin M., Primožič M., Knez Ž., 2007, Supercritical fluids as solvent for enzymatic reactions, 54, 667-677.

Hanefeld U., 2009, Modern biocatalysis, Immobilization as a tool for improving enzymes. Wiley-VCH, Weinheim, Germany.

Sheldon R.A., Schoevaart R., Van Lagen L.M., 2005, Cross-linked enzyme aggregates (CLEAs): A novel and versatile method for enzyme immobilization (a review). Biocatalysis and biotransformation. 23 (3/4), 141-147.

Sheldon R.A., 2007, Biocatalysis in the pharmaceutical and biotechnology industries, Immobilization of enzymes as cross-linked enzyme aggregates: A simple method for improving performance. CRC Press, Boca Raton, United States of America.

Zhou Y.T., Su S.N., Song M.M., Nie H.L., Zhu L.M., 2009, Improving the stability of cellulose immobilization, Bioinformatics and biomedical engineering, 1-4.