Recovery of a Recombinant Thermostable Endoglucanase from *E. Coli* Using Supercritical Carbon Dioxide Cell Disruption

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For the production of thermostable endoglucanase from *Clostridium thermocellulum* the celC gene was cloned into *Escherichia coli* BL 21 (DE3) host. The recombinant *E. coli* was grown in shake flask cultures. The intracellular recombinant protein was extracted from the cells after applying supercritical CO_2 cell disruption. The supercritical CO_2 cell disintegration was optimized and then compared to the traditional ultrasonic cell disruption technique. With the supercritical cell disruption the cellulase recovery was approximately 17 % lower than that of obtained with sonication.

Keywords:
Cell disintegration, *Clostridium thermocellum*, recombinant *E. coli*, supercritical carbon dioxide, sonication, thermostable endoglucanase

Introduction

Most of the best cellulase producing fungal strains, such as *Trichoderma* and *Aspergillus*, are mesophile organisms. The temperature optimum of these cellulases is around 50 °C. Therefore their application is limited. However, in certain technologies thermostable cellulase enzymes are needed. There is a particular interest for thermostable cellulases in the bioconversion of lignocellulosic raw materials into fuel hydrogen, where hyperthermophile bacteria are used for the production of hydrogen from monomeric sugars.[1] Another area of interest is the utilization of enzymes in the production of recycled paper. Using endoglucanases for the treatment of secondary fibers, better dewatering properties of the pulp can be obtained, which has several positive effects. For instance, the quality of the paper and the productivity of the paper machine can be increased.[2,3] Thermostable cellulases are also often used as additives to washing powder.[4,5] To overcome the problems often associated with anaerobic high temperature fermentations and to increase yield and productivity, cloning the particular gene from *Clostridium* to *E. coli*, seems to be advantageous. In the present study, *C. thermocellum* celC gene was cloned in *E. coli* BL 21 (DE3) containing the pET 21c vector.[6] However, using the construction described above the synthesized recombinant protein is not excreted to the culture medium. In order to recover the product, the cells have to be disintegrated and the enzymes have to be separated from the cell debris. One way of performing the cell disruption is using supercritical carbon dioxide. The supercritical carbon dioxide is extremely diffusible, therefore, it can penetrate into the cells. After the cells are saturated with carbon dioxide applying a sudden pressure drop, the lipid components of the cell wall are solubilized, and the cells are disintegrated due to the expansion of the CO_2.[7] The main advantages of this method are that the carbon dioxide applied is considered environmentally friendly, nonflammable and inexpensive. The most important aspect of supercritical CO_2 cell disruption is that the proteins are left intact. The technique is relatively simple, and compared to sonication, can easily be scaled up to industrial scale. There are only a few papers published on supercritical cell disintegration. For yeast cell disintegration longer (5 hours), while for bacteria considerably shorter (1 hour), residence times were found to be optimal.[8,9] For the effective treatment of both types of microorganisms, a very high pressure, 350 bars, was recommended. The third important factor of the supercritical cell disruption method is the temperature. At higher temperatures, the denaturation of the enzymes to be recovered was observed, and at lower temperatures higher yields were obtained.[8,9,10] In the present study, the applicability of the supercritical CO_2 cell disruption was examined for the recovery of a re-

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combinant thermostable endoglucanase from *E. coli*. The influence of three factors i.e. carbon dioxide temperature, CO_2 pressure, and residence time, on the enzyme recovery was studied. The supercritical cell disintegration was compared with sonication, which is a generally used method for cell disruption in laboratory scale. The results were statistically analyzed using commercially available software, Statistica for Windows.

Materials and methods

Bacterial strains and plasmids

Escherichia coli BL 21 (DE3) was obtained from the strain collection of the Hungarian Academy of Science, Institute of Enzymology.[6] The gene fragment encoding the thermostable cel*C* gene of *C. thermocellum* was cloned in the pET21c (Novagen, USA) expression vector, containing the T7 promoter and introduced to the host strain. The *E. coli* contained a modified lac operon encoding the T7 RNA polymerase. In the presence of isopropil-thiogalactosid (IPTG) inducer, the T7 RNA polymerase was synthesized and the recombinant protein was produced. *E. coli* BL 21 (DE3) was maintained on Luria-Bertani (LB) medium containing 100 μg mL^{-1} ampicillin.

Cultivation conditions

In all cases, *E. coli* was cultivated in LB medium supplemented with 100 μg mL^{-1} ampicillin. For the preparation of the starter culture, a single colony was transferred into a 100 mL E-flask containing 20 mL culture medium. The inoculum was thermostated in a rotary shaker at 30 °C and 350 rpm for 12 hours. The bacterium suspension obtained was used to initiate growth in 750 mL E-flask containing 150 mL culture medium. The grow up of culture was carried out in rotary shaker incubator at 30 °C and 350 rpm for 12–14 hours until 0.8 – 1.0 optical density (OD_{600}) was reached. After the addition of IPTG inducer in a concentration of 0.5 mmol L^{-1} (1st – 3rd batches) or 1.0 mmol L^{-1} (4th batch) the incubation was continued for another 3.5 hours. The fermentation broth was harvested and the cell suspension was centrifuged at 10 000 rpm for 10 minutes. The supernatant was removed and the biomass was collected. The dry mass of the biomass was around w = 6.5 %.

Cell disintegration with sonication

A gram of wet *E. coli* cells was resuspended in 40 mL of phosphate buffer (0.05 mol L^{-1}, pH 7.2). The cell disintegration was carried out using an MSE (PG-1533, 12–77) sonicator. The amplitude of the ultrasound was 29 μm. Prior to the experiments the sonication was optimized regarding residence time (data not shown). Maximum enzyme yield was reached after 27 minutes of residence time, which was therefore applied in all experiments.

Cell disintegration with supercritical carbon dioxide

Supercritical CO_2 cell disruption was carried out in a high-pressure equipment (Figure 1). The reactor vessel had an inner volume of 25 ml. Approximately 0.2 – 1.0 g wet *E. coli* cells were transferred onto an inert carrier (non-cellulosic material) and then placed into the reactor vessel. The vessel was filled with the supercritical CO_2 at different pressures (120 – 250 bars) and temperatures (32 – 45 °C). After 5 – 60 minutes of residence time the vessel was rapidly depressurized. Food grade CO_2 was supplied by Messer Ltd. of 99.5 % purity. The disintegrated cells were collected and were resuspended in 40 mL phosphate buffer (0.05 mol L^{-1}, pH 7.2).

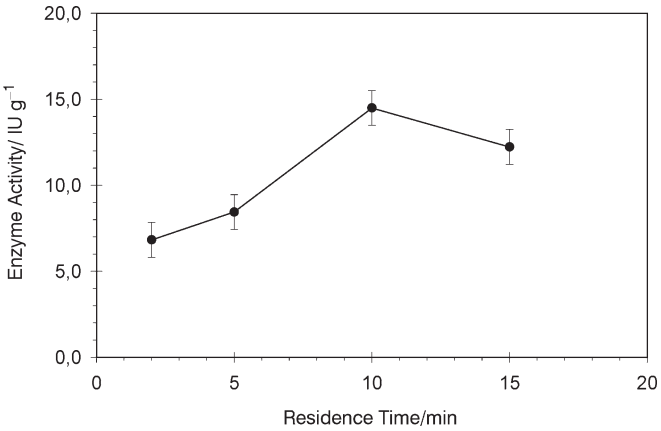

Fig. 1 – *The schematic flow sheet of supercritical cell disruption unit (1: CO_2 storage vessel, 2: cooler, 3: pump, 4: heat exchanger, 5: reactor, a – c: valves)*

Analytical methods

The suspensions obtained from, both, sonication and supercritical cell disruption, were centrifuged at 12 000 rpm for 10 minutes. The supernatants were collected and endoglucanase activities were determined.

The endoglucanase activity was measured using a modified Berghem method.[11] Instead of the substrate analogue of β-glucosidase, 4-nitro-phenyl-β-D-cellobioside (pNPC) was used, which facilitated the selective measurement of the endoglucanase activity. After incubation of the enzyme with pNPC at 50 °C for 10 minutes, the enzymatic reaction was terminated with addition of 2 mL 1 mol L^{-1} Na_2CO_3 solution. After dilution with 10 ml of distilled water the absorbance was measured at 410 nm.

Results and discussion

The effect of temperature on supercritical cell disintegration

In a set of experiments, the effect of supercritical CO_2 temperature on the enzyme recovery, was examined. Two different temperatures, 33 °C and 43 °C, were applied while the residence time, 37.5 minutes, and pressure, 150 bars, were kept at constant values. The results obtained are summarized in Table 1.

Table 1 – *The effect of CO_2 temperature on the enzyme recovery in supercritical cell disruption*

Temperature	33 °C	43 °C
Enzyme Recovery, IU g^{-1}	20.95	4.04
Number of repetitions	2	3
Standard Deviation, IU g^{-1}	1.33	0.80
Combined Standard Deviation, IU g^{-1}	1.01	

The temperature had significant effect on enzyme yields. It can been seen in Table 1, that at the lower temperatures about 5 times higher enzyme activities were measured compared to the results obtained at the higher temperature. A plausible explanation to the observed outcome of the experiments is that, although, the enzyme is a thermostable protein under these experimental conditions it shows thermal sensitivity in presence of carbon dioxide. Due to the results of this experimental setup, in the further experiments 32 °C operating temperature has been chosen. The critical temperature of CO_2 is 31.3 °C. Although, the chosen temperature is rather close to the critical temperature, previous experiences with the equipment used had shown that it could be operated safely at 32 °C.

Effects of pressure and residence time on supercritical cell disintegration

The effect of residence time (t_r) and pressure (p) on the enzyme recovery, has been examined, using a 2^2 full factorial design. The temperature was kept constant at 32 °C. One measurement was also performed in the center point. The results of the experiments are shown in Table 2.

Statistical analysis of the results was performed by fitting Equation 1 to the experimental response data i.e. enzyme recovery (y).

$$y = a_0 + a_1 \times p + a_2 \times t_r + a_{12} \times p \times t_r \quad (1)$$

Table 2 – *The results of factorial design*

Pressure/bar	Residence Time/min	Enzyme Activity/IU g^{-1}
250	60	16.08
100	60	12.54
250	15	24.28
100	15	10.08
175	37.5	18.77

The same standard deviation was used for the statistical evaluation of the results as previously (Table 1). T-test was performed to determine which factor has a significant effect on the enzyme recovery. As it can be seen on the Pareto chart of effects (Figure 2), the pressure and the interaction were found to have significant effect on the enzyme recovery. The fitted surface is plotted in Figure 3. The coefficient of pressure is positive, therefore, higher pressure will result in a better enzyme recovery. The effect of residence time was found to be insignificant, however, the combination of residence time and pressure (interaction) had a negative effect on the enzyme recovery. The best enzyme recovery was obtained at the highest operating pressure and at the shortest residence time. The 2^2 full factorial designs allow only linear model, therefore, curvature check was carried out to make sure the adequacy of the model. The curvature of the model was not significant at 0.05 confidence level thus the model was found adequate (data not shown).

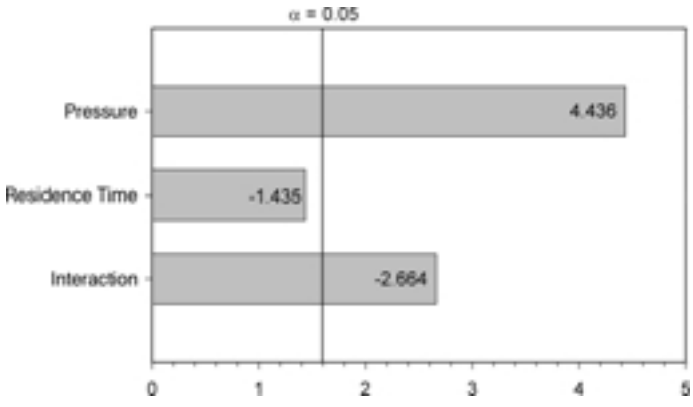

Fig. 2 – *Pareto chart of effects of factorial design*

To make sure the role of residence time, experiments were performed at 32 °C and 250 bar CO_2 pressure, where the residence time was varied between 2 and 15 minutes. At each experimental point, two parallel runs were carried out. As it is

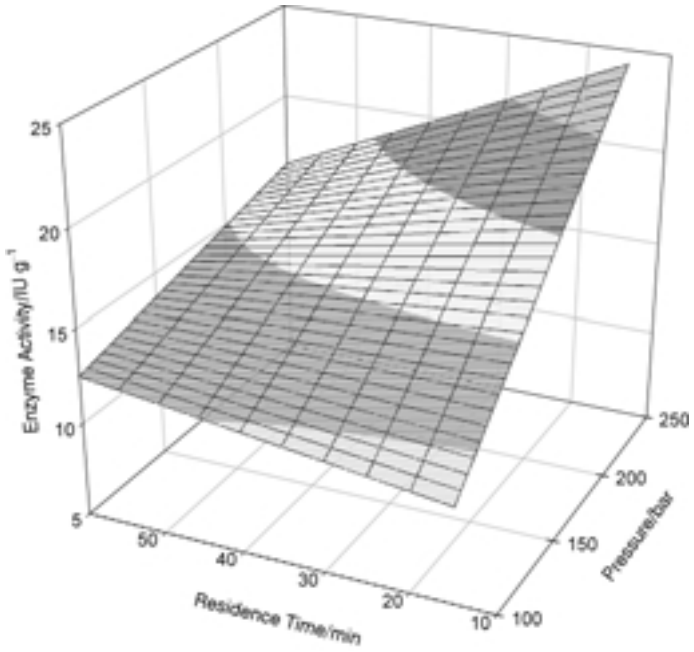

Fig. 3 – *The fitted response surface of factorial design*

shown in Figure 4 the enzyme recovery increased with treatment time up to 10 minutes, after which the amount of recovered enzyme was slightly decreased. This is in good agreement with the results obtained in the factorial design, where it was shown that in the range of 10 to 60 minutes, the treatment time had no effect at all.

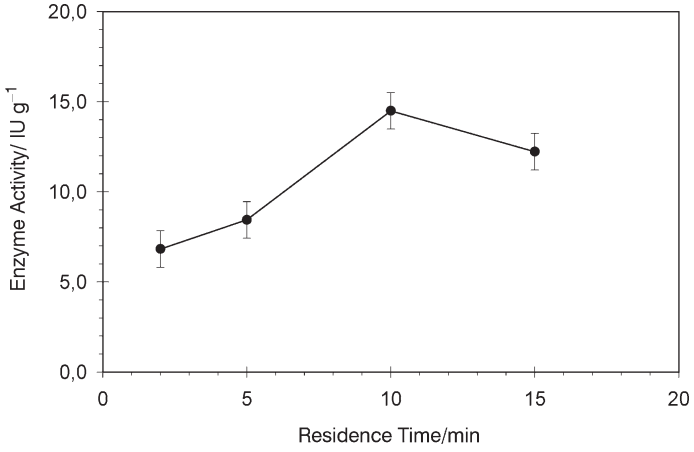

Fig. 4 – *The enzyme recovery as the function of residence time*

Comparison of cell disintegration methods

E. coli cells were also disintegrated with the traditional sonication technique under optimal conditions. The enzyme recovery was 44.7 IU g^{-1} cells, which was 17 % higher when compared to supercritical CO_2 disintegration under optimal conditions. However, the sonication cannot be scaled up, therefore supercritical cell disintegration is a promising method for industrial applications.

Conclusions

E. coli cells can be successfully disintegrated with supercritical carbon dioxide. Within the examined range the optimal condition was found at 32 °C, 250 bar and 10 minutes of residence time. The supercritical disintegration method was found 17 % less effective than the sonication method.

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