Examination of Cellulase Enzyme Production by *Trichoderma Reesei* Rut C30 Using Supercritical Carbon Dioxide Cell Disruption

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Cellulase enzyme production by *Trichoderma reesei* RUT C30 was examined on two different carbon sources, delignified pine pulp (Solka Floc, SF) and glucose. In order to determine the total amount of enzymes produced, besides measuring the extracellular cellulase and β-glucosidase activities, intracellular enzyme activities were also determined. Cell disruption was performed using supercritical carbon dioxide.

Cellulase activity could be detected only on SF, which is an inducer of cellulase production, whereas equal amounts of β-glucosidase activity could be observed on both carbon sources. When examining the „total" (extra and intracellular) cellulase and β-glucosidase activities achieved on SF carbon source, a ratio of 1:1.14 was found. This means that in the cell an optimal ratio of enzymes is produced but β-glucosidase is partly cellbound. The results also emphasise the difference in the production of β-glucosidase and the other components of the cellulase enzyme complex, that is, while the endo- and exoglucanases are inductive enzymes, β-glucosidase enzyme is produced constitutively.

Keywords:
Cellulase, *Trichoderma*, supercritical, carbon dioxide, disruption

Introduction

The partial or total hydrolysis of cellulose fibers using cellulases is a procedure applied in a number of industries. Nowadays the most widespread applications of cellulase enzymes are in the textile industry, where the enzymes are used in the stone washing of jeans and in the finishing of cotton fabrics.[1,2] Another significant user is the pulp and paper industry, where cellulases together with hemicellulases can be applied successfully, to improve the drainage of secondary fibers. Cellulases and hemicellulases are also used in the deinking process.[3,4]

In the industrial cellulase production mostly a genetically modified filamentous fungus, *Trichoderma* is used. It is capable of producing the extracellular enzyme at a high level. The cellulase enzyme complex consists of three main components: endoglucanases (EC 3.2.1.4.), exoglucanases (EC 3.2.1.91.) and β-glucosidase (EC 3.2.1.21.). The individual components work in close synergy, supporting each other's functions during the hydrolysis of cellulose.[5] Although the fermentation broth contains each of the three components of the enzyme complex, the amount of β-glucosidase, which is necessary for the final step in the degradation of cellulose to glucose, is less than that required for its efficient operation.[6,7] Much research has been done to determinate the optimal enzymatic composition for the hydrolysis of various cellulose substrates. It can be stated that the ratio of enzymes is the most suitable when the cellulase activity measured against filter paper and the β-glucosidase activity shows a ratio of 1:1.[8]

As a considerable part of the produced, β-glucosidase enzyme stays in the intracellular space 22, the composition of the enzyme complex in the fermentation broth is not effective enough in the hydrolysis of crystalline cellulose, thus it needs to be supplemented with β-glucosidase. A supplementation can be performed with β-glucosidase produced by other microbial strains e.g.: *Aspergillus* or else β-glucosidase can be obtained by the disruption of *Trichoderma* cells.

Various cell disruption techniques have been developed over the years but only a few are available on a large scale. Currently, the use of mechanical methods is the most widespread in industry, although a number of non-mechanical methods, especially enzymatic lysis, have become the focus of attention.[9] The disadvantage of the majority of cell disruption methods is that besides cell disruption, they also result in the degradation and denaturation of smaller/larger proportions of the proteins.

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Supercritical carbon dioxide cell disruption is a potential method for the extraction of sensitive biological materials due to a number of advantageous reasons compared to other techniques.

Carbon dioxide reaches a supercritical state at a temperature of 31.1 °C and above a pressure of 73.8 bar, and due to this, its properties change: it is characterized by "gas-like" mass transfer and liquid dissolving power. Its dissolving power is dependent on pressure and temperature. The solvent can be removed from the reaction media without any residue by only changing the temperature and pressure below the supercritical values of carbon dioxide. It is inexpensive, non-flammable and non-toxic. Due to these favourable properties, it has been applied in extraction processes[10–12] and as the solvent of enzymatic reactions.[13] It has been playing an increasingly important role in the food industry, the pharmaceutical industry, and in the field of environmental protection.[11–13]

The characteristics mentioned above make it suitable for use as a "tool" in cell disruption. Due to its high diffusibility and its apolarity, the supercritical fluid easily dissolves into the cell membrane, diffuses through it, and fills the intracellular space as well. Due to the decrease in pressure, it returns to a gaseous state, thus requiring larger volume of space, which tears the cells apart. Enzymes do not lose their activity in media filled with supercritical carbon dioxide, and the relatively low temperature is particularly advantageous for them. Furthermore, it can be assumed that as a result of the dissolved carbon dioxide inside the cell, the pH decreases, thus proteases will be inactivated, becoming incapable of degrading the rest of the proteins.[14] The only step that could lead to the inactivation of the enzymes is the decrease in pressure. The expansion of the carbon dioxide dissolved in the micro-aqueous layer around the enzyme can modify the enzyme structure to such an extent that it could become inactive. The extent of inactivation depends mostly on the pressure applied, the rate of pressure decrease, and the strength of the enzyme structure.[15] Therefore, the loss can be minimized or eliminated by the selection of suitable parameters. The cell disruption method is remarkably simple. With the increase of pressure, supercritical carbon dioxide enters the cells, and then due to a sudden decrease of pressure, it departs in a gaseous state, leaving disrupted cells in the holder. The efficiency of the method varies in various cells;[16,17] the presence of a cell wall and the characteristics of the cell surface have a strong influence.

The stability of cellulases in supercritical carbon dioxide has been investigated by *Zheng* and *Tsao*.[15] Based on their data it can be concluded that the enzyme stability in supercritical carbon dioxide is high and the enzyme structure does not suffer any damage when the pressure is decreased at a slow rate. Based on this knowledge, we examined the disruptability of *Trichoderma reesei* RUT C30 filamentous fungus, using supercritical carbon dioxide. Cellulase and β-glucosidase activities were measured in, both, extra and intracellular places in order to determine the ratio of the activities in the produced enzyme complex.

Trichoderma cultivation was performed on Solka Floc (delignified pine pulp) and glucose carbon sources. While the former is non-soluble and its degradation products induce enzyme synthesis, glucose is an easily metabolized carbon source, but not an inducer of the enzyme production.[18]

Materials and methods

Microorganism

Trichoderma reesei RUT C30 was obtained from the strain collection of the Department of Agricultural Chemical Technology, Budapest University of Technology and Economics, Hungary and maintained on potato-glucose-peptone agar at 30 °C.

Cultivation conditions

The fermentation was performed in a Biostat U30 laboratory fermentor. The fermentor´s working volume was 20 L.

The fermentation parameters: temperature: 28 °C, pH 5.7 (adjusted with 10 % H_2SO_4 and 10 % NaOH), stirring speed: 250 rpm, airflow: 1 vvm.

The medium was prepared based on Mandels´,[19] the mass concentration of carbon sources, Solka Floc (SF) and glucose, was 10 g L^{-1}.

Inoculum was prepared in 750 mL E-flasks, using 150 mL/flask medium (Mandels´). 14 days old agar slants were used. The flasks were aerated on a rotary shaker (350 rpm), the temperature was 30 °C. The inoculum was grown for 4 days in the case of SF and for 3 days in the case of glucose carbon source.

10 % inoculum was used in the fermentation experiments. 200–200 mL samples were taken 3 times a day. After sampling a known amount, the fermentation broth was centrifuged at 10 000 rpm for 10 min.

The supernatant was kept for enzyme activity determinations and the precipitate was washed 2 times in distilled water. Dry mass determination and cell disruption were performed from the washed cell samples.

Supercritical cell disruption

Cell disruption was performed in a unique batch operated, high pressure reactor. The inner volume of the reactor vessel was 25 mL. 1 g *Trichoderma reesei* cell was put into the reactor vessel. It was filled with 100 bar, 40 °C supercritical carbon dioxide. When reaching the end of the planned residence time the excess pressure was gradually released by opening the expansion valve.

Cell debris was washed out with 20 mL citrate buffer (0.05 mol L^{-1}, pH 4.8) from the reactor vessel.

The suspension was separated in a centrifuge at 10 000 rpm, the enzyme activities were determined in, both, the supernatant and the cell debris fraction which has been washed twice previously in distilled water.

Analytical methods

Cellulase activity was determined using FPU measurements.[20] Filter paper activity (FPU) was determined by incubating 1.5 mL of suitably diluted supernatant with 50 mg (1 cm × 6 cm) of Whatman No. 1 filter paper in citrate buffer (0.05 mol L^{-1}, pH 4.8). After 60 minutes of incubation at 50 °C the reducing sugars were measured in the supernatant by DNS method, using glucose as a standard. The activity was calculated in IU mL^{-1} (μmol mL^{-1} min^{-1}).

β-glucosidase activity was determined by incubating 100 μL of supernatant with 1 mL of *p*-NPG (para-nitrophenyl-β-D-glycopyranoside, 5 mmol L^{-1}). After 10 min of incubation at 50 °C the reaction was terminated by adding 2 mL of 1 mol L^{-1} Na_2CO_3 solution. After being diluted with 10 mL of distilled water the absorbance was measured at 400 nm.

Enzyme activities were determined, both, from the supernatant of the fermentation broth and the supernatant of the cell debris suspension. The measurements were also performed with the cell debris fraction, which was suspended in 20 mL distilled water.

Calculations:

Extracellular enzymatic activity (IU_e):

Enzyme activity (IU mL^{-1}) (from supernatant of fermentation broth) × Σ fermentation broth supernatant (mL) (in fermentor)

Intracellular enzymatic activity (IU_i):

Enzyme activity (IU mL^{-1}) (from supernatant suspension of cell debris) × quantity of suspension (mL) / disintegrated cell mass (g) × Σ mass of cells in fermentation broth (g) (in fermentor)

Results and discussion

The effect of residence time on supercritical cell disintegration

The aim of these experiments was to determine the necessary residence time in the high pressure reactor using constant temperature (40 °C) and pressure (100 bar) based on preliminary experiments (data are not shown).

Cells originating from a 50 h sample produced on SF were used in the experiments. 15, 30, 45 and 60 min of residence time were examined (Table 1).

Table 1 – *Cellulase activities after cell disruption under various residence times (100 bar, 40 °C)*

Residence time, τ/min	$a_{r\ FPU}$/IU g^{-1}
60	7.81
45	8.05
30	7.95
15	8.14

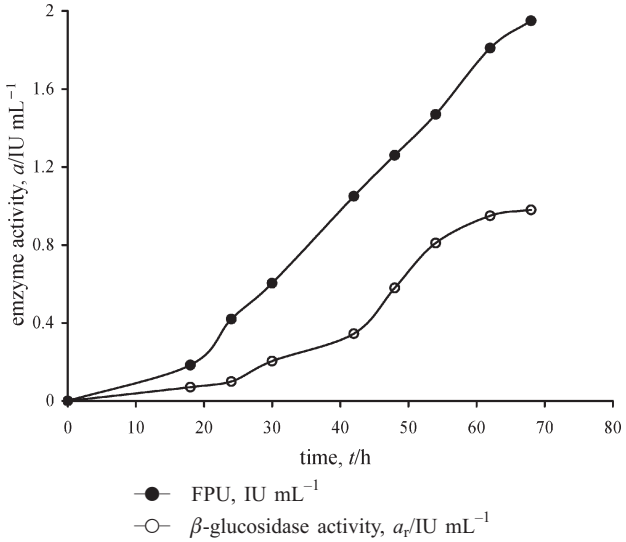
It can be clearly seen that 15 min of residence time is sufficient for cell disruption to take place under the above mentioned conditions. Results also show that the resistance of *Trichoderma* cells to supercritical carbon dioxide is not considerable, particularly if it is compared to the disruption of *Saccharomyces cerevisiae* cells, where 2 h of incubation time at 35 °C temperature and 210 bar pressure is needed to disrupt 80 % of cells.[21]

Cellulase enzyme production using Solka Floc carbon source

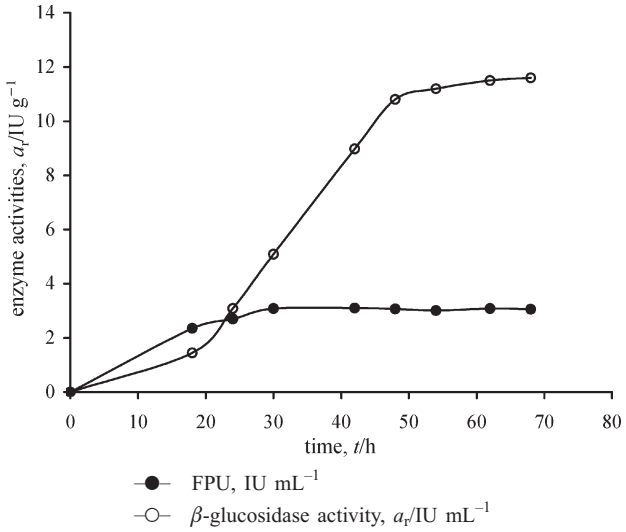
Figure 1 shows the cellulase and β-glucosidase activities measured during the fermentation in the fermentation broth's supernatant. It can be clearly seen that compared to the cellulase activity, β-glucosidase appears after a longer lag phase and at a slower rate in the extracellular space. At the end of fermentation the ratio of the cellulase and β-glucosidase activities is 1:0.5 which does not reach the 1:1 ratio optimal for degrading cellulose to glucose.

Figure 2 shows the enzyme activities inside the cell. The intracellular cellulase activity reaches a constant value within a relatively short period of time (approx. 24 h). This time roughly coincides with the time when enzyme production reaches an exponential phase outside the cell.

The intensive production of β-glucosidase in the extracellular space apparently starts after reaching the constant internal activity level. The β-glucosidase enzyme amount increases within the cell



F i g . 1 – *Cellulase production on SF by Trichoderma reesei RUT C30; Enzyme activities in the supernatant of fermentation broth.*



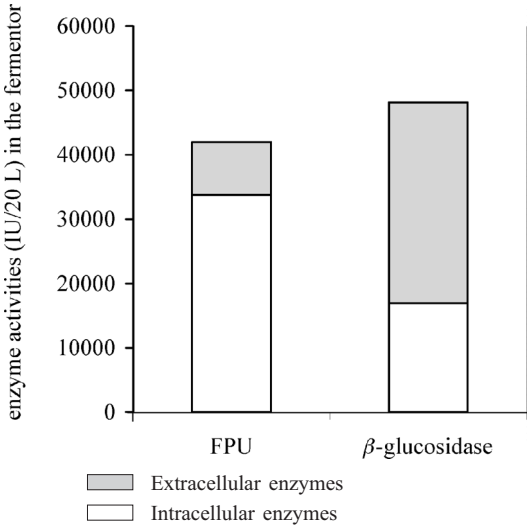
F i g . 2 – *Intracellular cellulase production on SF carbon source by Trichoderma reesei RUT C30*

for approximately 48 h, then remains at a constant value. Though it takes a longer time to reach this constant value than in the case of the cellulase, the value of enzyme activity is much higher, approximately 11.5 IU g^{-1} cell. The activity is almost quadruple.

The intracellular enzyme activity values derive from the supernatant of the cell debris suspension. However, activity measurements were also performed from the cell debris fraction but there was no measurable enzyme activity.

Total (intra and extracellular) enzyme activities are shown in Figure 3. Data were collected from the last sample of fermentation (67 h).

If we take into account the enzymes in the intracellular space as well, we get the enzyme profile necessary for the efficient degradation of cellulose into glucose. The ratio of FPU/β-glucosidase in the whole broth (including the mycelium) is: 41980/48160, i.e.: 1/1.14. While the β-glucosidase activity in the supernatant of the fermentation broth is half of the cellulase activity, in the intracellular space the amount of the former is four times that of the cellulase activity. These results together with previous publications[23] indicate that just a part of β-glucosidase is extracellular, the other part of enzyme is either cell-wall-bound or intracellular protein. According to our experiments it can also be established that the carbon dioxide in supercritical state not only tears the cell wall and membrane during expansion but it is also able to detach the enzyme from the cell debris.



F i g . 3 – *Total enzyme activities at the end of the fermentation on SF carbon source*

Cellulase enzyme production using Glucose carbon source

The cellulase activity measured in the fermentation broth on glucose carbon source – in accordance with the literature and our expectations – is practically zero. Endo- and exoglucanases are inducible enzymes, thus their production is impossible if their inductors are not present in the medium. The β-glucosidase enzyme appeared in measurable quantities by the 60th hour of the fermentation. In the 72nd hour its activity reached 0.24 IU mL^{-1}.

Figure 4 shows the intracellular enzyme activity values. The enzyme activities within the cell also show a saturation curve. The cellulase activity is practically negligible, while the β-glucosidase enzyme reaches a value of 11.5 IU g^{-1} activity in the cell. This interior value is obtained around the 60th h, approximately at the same time as it appears in the extracellular space. Since intracellular β-glucosidase appears in the cells from the 40th h, when the

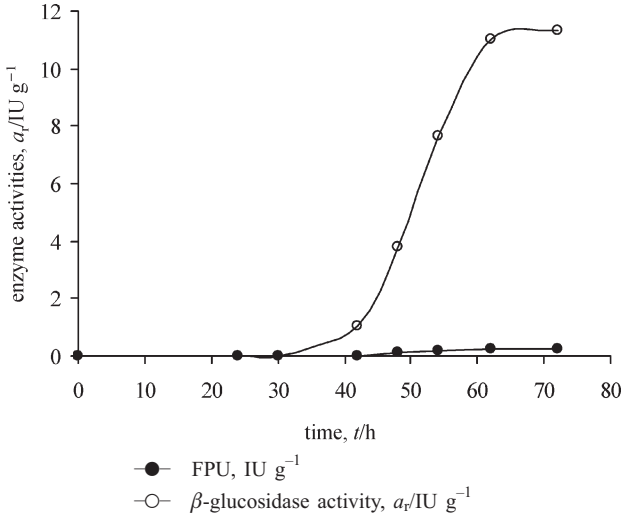


Fig. 4 – *Enzyme activities inside the cells on Glucose carbon source*

glucose concentration is practically zero in the culture medium, it can be suggested that the enzyme production was under catabolite repression until then. Unlike the other cellulase compounds, this enzyme is produced constitutively. The probable explanation of the appearance of exracellular β-glucosidase is the cell autolysis, which is considerable by this time, thus the intracellular cell-matter is discharged in the extracellular space.

Examining the total amounts of β-glucosidase produced on glucose carbon source, we find a value of approximately 55000 IU / 20 L. Although practically no cellulase activity was detected, an equivalent production of the β-glucosidase enzyme can be observed as on SF. While in this case the intracellular enzyme was 90 % of the total amount, it was 60 % on Solka Floc.

Based on the amounts of the measured β-glucosidase, it can be stated that the enzyme is produced constitutively, unlike the other components of the cellulase enzyme complex. On the basis of the measurements, it can be concluded that enzyme production on glucose is initiated at a slower rate and it appears in smaller amounts in the extracellular space than observed on the cellulose carbon source. In spite of this, the amount measured inside the cell is equivalent in both cases. This observation is in agreement with previous studies, which concluded that glucose does not have any influence on the production of the β-glucosidase enzyme, only on its location.[22]

In summary, the cell disruption performed with supercritical carbon dioxide in laboratory quantities proved to be efficient and executable. The results obtained under the selected conditions, 40 °C, 100 bar and 15 min of residence time, show that the *Trichoderma*'s resistance to supercritical carbon dioxide is not high.

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References

1. *Hemmpel, W. H.,* ITB Dyeing/Printing/Finishing **3** (1991) 5.
2. *Kumar, A., Yoon, M-Y., Puntell, C.,* Textile Chemist and Colorist **29** (1997) 37.
3. *Jokinen, O., Kettunen, J., Lepo, J., Niemi, T., Laine, J. E.* United States Pat. (1991) 5,068,009.
4. *Pommier, J. C., Goma, G., Fuentes, J. L., Rousset, C., Jokinen, O.,* Tappy J. **73** (1990) 197.
5. *Persson, I., Tjerneld, F., Hahn-Hägerdal, B.,* Process Biochemistry **26** (1991) 65.
6. *Allen, A., Sternberg, D.,* Biotechnol. Bioeng. Symp. **10** (1980) 189.
7. *Wood, T. M., McCrae, S. I.,* American Chemical Society 1981, 181.
8. *Joglekar, A. V., Karanth, N. G., Srikivasan, M. C.,* Enzyme Microbiol. Technol. **5** (1983) 25.
9. *Chisti, Y., Moo-Young, M.,* Enzyme Microbiol. Technol. **8** (1986) 194.
10. *Williams, D. F.,* Chem. Eng. Sci. **36** (1981) 1769.
11. *Smith, R. M., Burford, M. D.,* J. Chromatogr. **627** (1992) 255.
12. *Bertucco, A., Vetter, G. (Eds.)*: High Pressure Process Technology: Fundamentals and Applications, Elsevier, Amsterdam, 2001.
13. *Perrut, M.,* Chem. Biochem. Eng. Q. **8** (1) (1994) 25.
14. *Isenschmid, A., Marison, I. W., von Stockar, U.,* J. Biotechnol. **39** (1995) 229.
15. *Zheng, Y., Tsao, G. T.,* Biotechnol. Lett. **18** (1996) 451.
16. *Lin, H., Chan, E., Chen, C., Chen, L.,* Biotechnol. Prog. **7** (1991) 201.
17. *Lin, H., Yang, Z., Chen, L.,* Biotechnol. Prog. **8** (1992) 165.
18. *Kubicek, C. P.,* Proceedings of the second TRICEL symposium on *Trichoderma reesei* cellulases and other hydrolyses, Espoo, Finland, 8 (1993) 181.
19. *Mandels, M., Weber, J.,* Adv. Chem. Ser. **95** (1969) 391.
20. *Mandels, M., Andreotti, R., Rooche, C.,* Biotechnol. Bioeng. Symp. **6** (1976) 21.
21. *Lin, H., Chan, E., Chen, C., Chen, L.,* Biotechnol. Prog. **7** (1991) 201.
22. *Umile, C., Kubicek, C. P.,* FEMS Microbiology Letters **34** (1986) 291.
23. *Chirico, Brown, R. D. Jr.,* Eur. J. Biochem **165** (1987) 333.