Effect of Supercritical Carbon Dioxide on Activity of Different Proteins Released from *Hortaea Werneckii*

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ABSTRACT

In this study, the suspension culture of *Hortaea werneckii* was incubated in supercritical carbon dioxide (SC CO_2) in order to use the enzymes from these fungi for biotransformations and compare their activity with the activity of purified commercial enzymes at the same conditions. *H. werneckii* cell suspension was treated in SC CO_2 at 35 °C and different pressures. The survival of *H. werneckii* cells was determined by measuring the optical density of the cell suspension. Additionally, the total protein concentration and absorbance of nucleic acids in the suspension were also determined. The interest was to excrete the intracellular enzyme α-amylase from *H. werneckii* and to keep the extracellular enzyme cellulase in the active form after the treatment of the cell suspension culture with SC CO_2. The activity of intracellular enzyme eliminated from *H. werneckii* cells and that of extracellular enzyme after the treatment of the cells with SC CO_2 was compared with the activity of commercial available enzymes. A high amount of proteins was released from *H. werneckii* cells during the SC CO_2 treatment. The residual activity of both examined enzymes decreased with prolonged treatment time of *H. werneckii* cell suspension in SC CO_2, but both could be still used as biocatalysts in this medium.

Key words: *H. werneckii*, supercritical carbon dioxide, cellulase, α-amilase.

INTRODUCTION

Hortaea werneckii is black yeast which belongs to the order *Dothideales*. Black yeasts are characterized by slowly expanding colonies. Their main features are polymorphism, meristematic growth, endoconidiation, frequently muriform cells, which develop by conversion from undifferentiated hyphae and thick, melanized cell walls. This morphological ecotype is important for their survival in different extreme environments [1]. *H. werneckii* is a very excellent adaptable fungi, it can grow without salt and at extremely high concentration of salt. *H. werneckii* was long known primarily as the etiological agent of human *tinea nigra*, a superficial infection of the human hand, particularly frequent in warmer areas of the world [1,2,3,4].

SC CO_2 is often discussed as an alternative, environmentally benign reaction medium for biocatalysis, due to its non-toxic and nonflammable nature, and its relatively low critical pressure and temperature (7.36 MPa, 31 °C) which allows preservation of thermally unstable compounds. Furthermore, SC CO_2 is climate neutral and inexpensive because the CO_2 used is

a byproduct of industrial processes [5]. Exposure time, pressure, temperature, initial number of cells, pressure cycling, initial pH of medium, water activity, cell growth phase or age, species of microorganisms and type of treatment system all might have effects on microbial inactivation by SC CO_2 [6].

MATERIAL AND METHOD

Microbial strains and growth conditions

Culture of *H. werneckii* was maintained on meat extract agar (MEA) slants at 25 °C. *H. werneckii* was inoculated in a prepared sterile meat extract broth, mixed by swirling and incubated at 25 °C with moderate shaking for descrambling. Before each experiments the culture suspension were freshly prepared by the same procedure.

Exposure of cell suspension to SC CO_2

The sensitivity of the *H. werneckii* cell suspension on SC CO_2 treatment was determined under varying pressures (10 and 30 MPa) and constant temperature 35 °C, for different treatment durations (1-24 h). Experiments were performed in a 70 mL high-pressure batch reactor. The sterile ampoule was filled with freshly prepared *H. werneckii* cell suspension and then it was placed into reactor. When the temperature of the system reached a set temperature, the reactor was supplemented to the desired pressure with cooled CO_2.

Commercially available enzymes, cellulases (cellulase from *H. insolens* and cellulase from *T. reesei*, Cellusoft L) and α-amylase (α-amylase from *A. oryzae*) were exposed to SC CO_2 at identical conditions as cell suspension of *H. werneckii*, using same procedure for exposure as described above.

Measurement of UV-absorbing substances

Cell suspension of *H. werneckii* was centrifuged at 10000 rpm after incubation in SC CO_2. Concentration of nucleic acid, cellulase and α-amilase were detected using UV-Vis spectrophotometer at a wavelength of 260 nm, 340 nm and of 595 nm before and after incubation of the black yeast culture in SC CO_2. The proteins concentration in cell suspension *H. werneckii* was determined by the Bradford method [7]. The survival of *H. werneckii* cells was determined by measuring the optical density (600 nm) of the cell suspension.

RESULTS AND DISCUSSION

Survival of *H. werneckii* after the treatment in SC CO_2

Survival of *H. werneckii* cells in suspension exposed to SC CO_2 was determined.

Survival of *H. werneckii* was monitored spectrophotometrically by measuring optical density of the cultures at 600 nm.

The Fig. 1 shows that after one hour incubation of cell suspension in SC CO_2 at 100 and 300 bar the survival of cells significantly decreased. The survival of cells after 1h of incubation at 100 and 300 bar was only 14% and 12%.

After 24 h incubation of cell suspension in SC CO_2, almost all cells of *H. werneckii* were died. The culture of *H. werneckii* is not as resistant to high pressure as to high salt concentration.

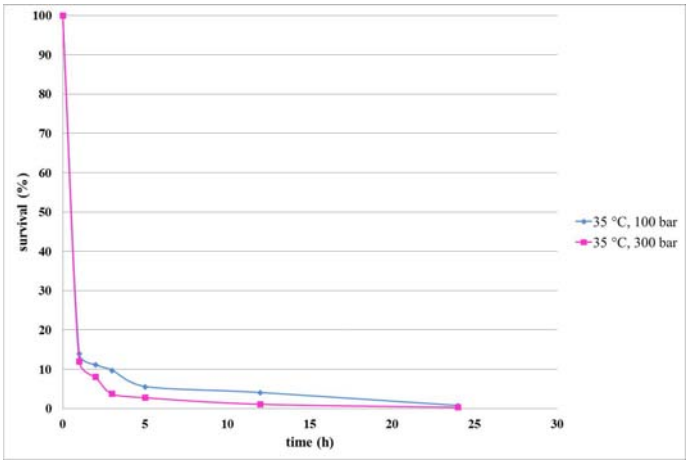

Figure 1: Survival of *H. werneckii* cells as a function of time, at 35 °C during SC CO_2 treatment.

Influence of SC CO_2 treatment on protein and nucleic acid concentration in suspension of *H. werneckii*

The cell materials, such as protein and nucleic acid, were released as a result of the SC CO_2 treatment. The treated cell suspension of *H. werneckii* was centrifuged 5 min at 10000 rpm. The contain of nucleic acid in supernatant was measured by the UV- vis spectrophotometer at 260 nm. The protein concentration in cell suspension was assayed by the Bradford method at the wavelength of 595 nm.

The protein concentration of the cell suspension *H. werneckii* after incubation in SC CO_2 was increased with increasing pressure and incubation time. High pressure led to permeabilization of cell walls and membranes. Intracellular proteins were extracted from the cells of *H. werneckii* with incubation in SC CO_2 and consequently, the total protein concentration in the suspension of *H. werneckii* culture was increased. The highest protein concentration in cell suspension of *H. werneckii* was detected after 24 h of incubation in SC CO_2 at 300 bar (Fig. 2).

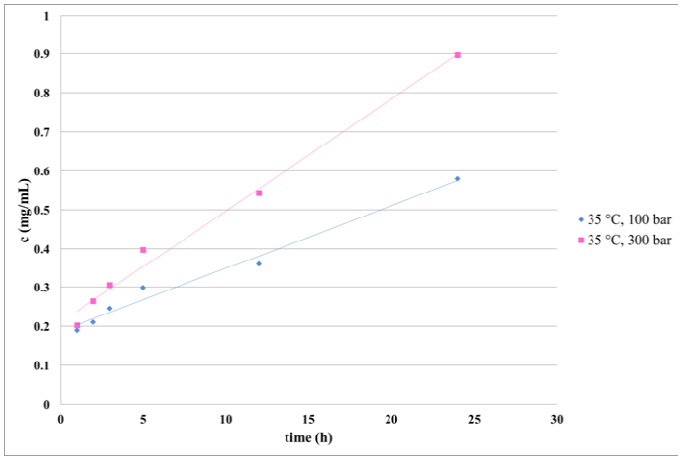

Figure 2: Protein concentration in the cell suspension of *H. werneckii* after SC CO_2 treatment at 35 °C and different pressures.

The protein concentration increased linearly with increase in incubation time independence of pressure.

With increase in the incubation time, the absorbance of nucleic acids remains linear. The absorbance of nucleic acids in the cell suspension incubated at 300 bar is higher than in the suspension, which was incubated at 100 bar (Fig. 3). This was also expected because at higher pressure more cells were opened and more cellular material was released.

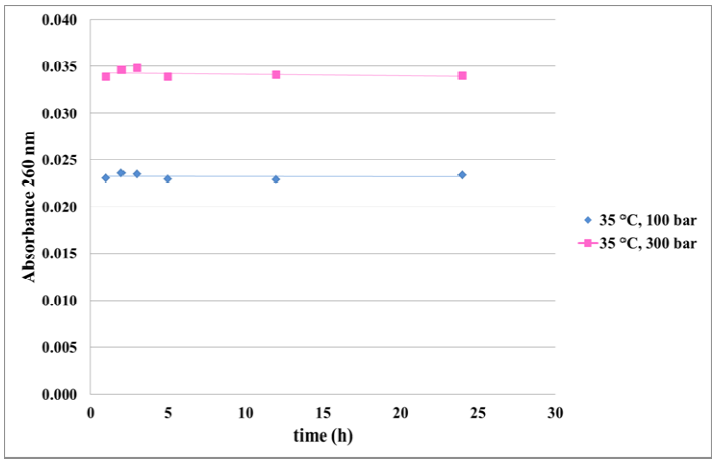

Figure 3: Absorbance of nucleic acids in the cell suspension of *H. werneckii* after SC CO_2 treatment at 35 °C and different pressures.

Activities of cellulases and α-amylases from *H. werneckii* cell suspension and of commercial available enzyme preparations

Activity of cellulases

The activity of cellulase and α-amylase in suspension of *H. werneckii* were measured on UV-Vis spectrophotometer before and after incubation in SC CO_2 at the wavelengths 340 nm and 595 nm. Cellulase is an extracellular enzyme which activity was determined in cell suspension of *H. werneckii* before incubation in SC CO_2. The highest activity of cellulase from *H. werneckii* after incubation of cell suspension in SC CO_2 was detected after 1 h exposure at 10 MPa (Fig. 4a). High pressures together with long incubation time in SC CO_2 led to deactivation of cellulase activity at both examined pressures.

For the comparison, cellulase from *T. reesei* (Cellusoft L) and pure cellulase from *H. insolens* were treated in SC CO_2 at the same conditions than *H. werneckii* cell suspension. Figs. 4b and 4c show the residual activity of cellulases from *H. insolens* and *T. reesei* (Cellusoft L) after incubation in SC CO_2. The maximum residual activities of commercial cellulases (Cellusoft L and enzyme from *H. insolens*) were reached after one-hour exposure in the SC CO_2 at 300 bar and were amounted to 135 % for Cellusoft L and to 206 % for cellulase from *H. insolens*. The highest activity of studied cellulases was detected for cellulase from *H. insolens*, because the cellulase from *H. insolens* was exposed to SC CO_2 in solid form. Cellulase from *H. werneckii* and cellulase from *T. reesei* have lower but very similar activities, because of decrease in pH due to SC CO_2 effect. It could be predicted that the activities of cellulases from *H. werneckii* and T. *reesei* exposed in powder form to SC CO_2, will be higher than those of liquid form.

With the increase of incubation time up to 24 h, the cellulase deactivated constantly (Fig. 4) irrespective of the cellulase origin. Cellulase activity is influenced by pressure, temperature and incubation time.

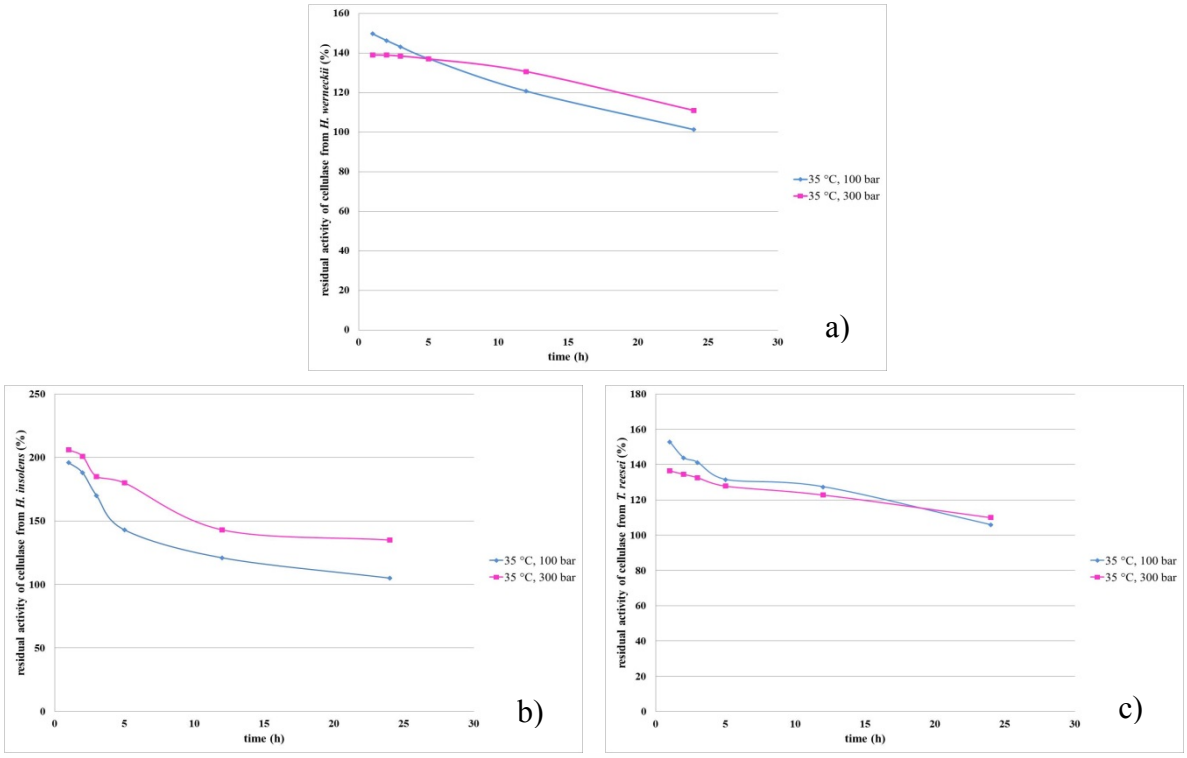

Figure 4: The residual activity of **a)** cellulase from *H. werneckii* cells, **b)** pure cellulase from *H. insolens* and **c)** cellulase from *T. reesei* (Cellusoft L) after incubation in SC CO_2 at 100, 300 bar and at 35 °C as a function of incubation time.

Activity of α-amylases

α-Amylase from *H. werneckii* is opposite to cellulase an intracellular enzyme. α-Amylase activity in cell suspension of *H. werneckii* was measured before incubation in SC CO_2. Low activity of α-amylase was detected. After incubation of the cell suspension in SC CO_2, the α-amylase activity was increased. The cells of *H. werneckii* in suspension were opened and the enzyme was eliminated from cells in the meat extract broth. The highest residual activity of α-amylase was achieved at 1h and 300 bar (Fig. 5a).
Longer exposure to SC CO_2 caused a decrease in α-amylase activity in *H. werneckii* cell suspension. To study the effect of SC CO_2 on activity of α-amylase from *A. oryzae* in the form of a purified powder, the enzyme was exposed to SC CO_2 at the same conditions as the cell suspension of *H. werneckii*.

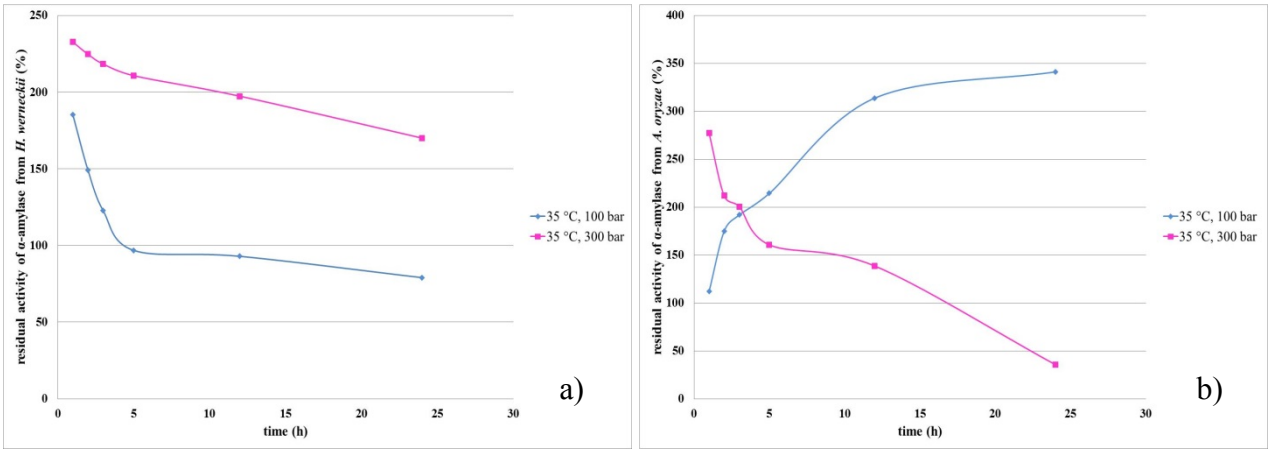

Figure 5: The residual activity of **a)** α-amylase from *H. werneckii* cells and **b)** α-amylase from *A. oryzae* after incubation in SC CO_2 at 100, 300 bar and at 35 °C as a function of incubation time.

The activity of α-amylase from *A. oryzae* after one hour of incubation in SC CO_2 was higher than the activity of nonincubated cellulase (Fig. 5b). When α-amylase from *A. oryzae* was incubated in SC CO_2 at 300 bar for one hour, a significant increase in its activity was observed but with longer incubation time the α-amylase activity started to decrease. On the contrary, the activity of α-amylase was increased with prolongation of incubation time, when the enzyme was exposed to SC CO_2 at 100 bar.

CONCLUSION

This paper examined the effect of pressure and temperature after exposure of cell suspension to SC CO_2 on the survival of *H. werneckii* cells and on enzyme activity of cellulase and α-amylase. From the *H. werneckii* cells during the SC CO_2 treatment a significantly high amount of proteins was released. It was found that the *H. werneckii* cells contain extracellular enzyme cellulase and intracellular enzyme α-amylase. High pressure together with long incubation times in SC CO_2 caused a decreasing of cellulase and α-amylase activity.

REFERENCES

[1] GUNDE-CIMERMAN, N., OREN, A., PLEMENITAŠ, A., Adaptation to life at high salt concentration in archaea, bacteria and eukarya, Springer, **2005**.
[2] GUNDE-CIMERMAN, N., PLEMENITAŠ, A., Reviews in Environmental Science and Biotechnology, Vol. 5, **2006**, p. 323.
[3] ABLIZ, P., FUKUSHIMA, K., TAKIZAWA, K., MIYAJI, M., NISHIMURA, K., Diagnostic Microbiology and Infectious Disease, Vol. 46, **2003**, p. 89.
[4] PLEMENITAŠ, A., VAUPOTIČ, T., LENASSI, M., KOGEJ, T., GUNDE-CIMERMAN, N., Studies in Mycology, Vol. 61, **2008**, p. 67.
[5] WIMMER, Z., ZAREVÚCKA, M., International Journal of Molecular Sciences, Vol. 11, **2010**, p. 233.
[6] LIAO, H., ZHANG, L., HU, X., LIAO, X., International Journal of Food Microbiology, Vol. 137, **2010**, p. 81.
[7] BRADFORD, M.M., Analytical Biochemistry, Vol. 72, **1976**, p. 248.