ACTIVITY OF ENYMES FROM *HORTAEA WERNECKII* IN SUPERCRITICAL CARBON DIOXIDE

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Abstract

Activity of enzymes from *Hortaea werneckii* in supercritical carbon dioxide is described. The suspension of *H. werneckii* was incubated in SC CO_2 in order to use the enzymes (cellulase, an extracellular protein, and α-amylase, an intracellular protein) from these fungi for biotransformation. The activity of both enzymes was tested to determine whether the enzymes were deactivated after being exposed to SC CO_2. *H. werneckii* was inoculated in liquid medium, mixed by swirling and incubated at 25 °C for 30 minutes with moderate shaking for descrambling. The suspension of the *H. werneckii* was incubated in SC CO_2. Experiments were performed in a 120 mL high-pressure batch reactor at 35°C, 100 and 300 bar and different incubation times (1-24 h) in SC CO_2. Nucleic acid concentration and the activity of cellulase and α-amylase were measured by UV-Vis spectrophotometer at 260 nm, 340 nm and 595 nm before and after incubation of the black yeast culture in SC CO_2. The protein concentration in the suspension of the cells was assayed by the Bradford method.

Introduction

Hortaea werneckii belongs to those yeast-like fungi, so-called black yeast, able to produce dark pigment inside and outside the cell wall. Black yeast is characterized by slowly expanding colonies [1]. The life cycle of *H. werneckii* includes yeast-like, hyphal and meristematic growth. Many studies have indicated a potential pathogenicity of *H. werneckii*, as in the genus *Exophiala* exist more human pathogenic fungi. In the past it was known as an etiological factor for skin disease tinea nigra in humans [2,3]. Cell walls of fungi are very important to maintain osmotic homeostasis of fungi. They also protect the cell against mechanical damage, e.g. from dehydration due to high salt concentrations in the environment. SC CO_2 is becoming an important commercial and industrial solvent due to its role in chemical extraction in addition to its low toxicity and environmental impact.
Carbon dioxide is a non-toxic and inexpensive gas. Furthermore, since the critical values of pressure and temperature are relatively low [4], the gas is relatively easy to handle under supercritical conditions. Its low critical temperature is only slightly above room temperature, so thermal degradation is not a problem when a process is operated around the critical temperature. Because of these benefits, CO_2 has been proposed for use in other biomaterial

applications such as incorporating bioactive ingredients into biodegradable polymers and producing enzyme particles [5,6]. Moreover, in the supercritical state, CO_2 has low viscosity ($3\text{-}7\times10^{-5}$ Nsm^{-2}) and zero surface tension, so it can quickly penetrate complex structures and porous materials [6]. When SC CO_2 is applied to microorganisms, microbial inactivation is affected by the effect of pressure temperature and exposure time. In general, microbial inactivation is accelerated with increasing SC CO_2 pressure and temperature. Higher pressure enhances CO_2 solubility, facilitating both acidification and cellular contact and the CO_2 also exhibits a higher solvating power [7].

2 Materials and method

2.1 Materials

H. werneckii was obtained from Department of Biology, Biotechnical Faculty (Ljubljana, Slovenia). Carbon dioxide 2.5 was supplied by Messer MG Ruše, Slovenia. Peptone from meat, sodium acetate, di-potassium hydrogen phosphate trihydrate and acetic acid were purchased from Merck (Darmstadt, Germany). D-(+)-Glucose, malt extract, sigmacell cellulose, Amylose-Remazol Briliant Blue R, agar, glucose assay reagent and sodium chloride were supplied from Sigma (Schnelldorf, Germany).

2.2 Microbial strains and media

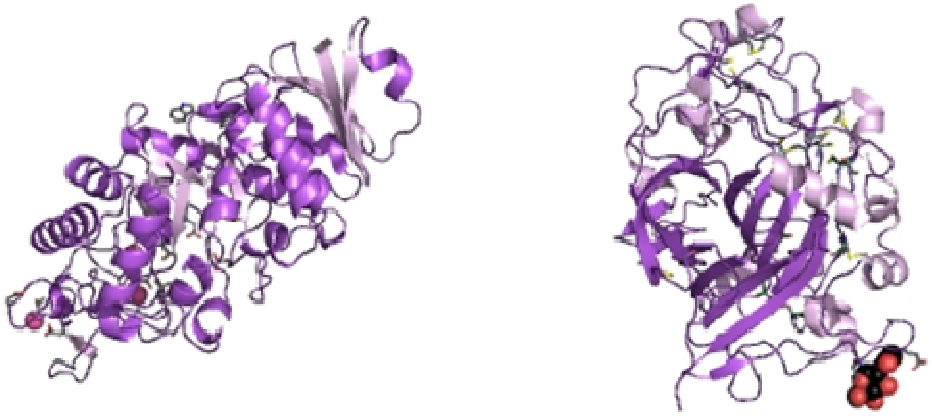
The black yeast *H. werneckii* was grown on meat extract agar (MEA) slants at 25 °C. A stock culture *H. werneckii* was inoculated in a prepared sterile meat extract broth, mixed by swirling and incubated at 25 °C with moderate shaking for descrambling. The final number of *H. werneckii* was 10^6 colony forming units (cfu/mL). The cultures used in all experiments were freshly prepared every day.

2.3 Pressure treatment

The suspension of the *H. werneckii* was incubated in SC CO_2. Experiments were performed in a 120 mL high-pressure batch reactor at the constant temperature (35 °C), different pressure (100 and 300 bar) and different incubation times (1-24 h) in SC CO_2. The sterile ampoule was filled with of freshly cell suspension of the *H. werneckii* and then it was placed into reactor. When the temperature of the SC CO_2 treatment system reached a set temperature, the reactor was supplemented to the desired pressure with CO_2.

2.4 UV- absorbing substances

Cell suspension of *H. werneckii* was centrifuged at 10000 rpm after incubation in SC CO_2. Nucleic acid concentration and the activity of cellulase and α-amylase were measured by UV-Vis spectrophotometer at 260 nm, 340 nm and 595 nm before and after incubation of the black yeast culture in SC CO_2. The protein concentration in the suspension of the cells was assayed by the Bradford method. Figure 1 shows enzyme of α-amylase from *Hordeum vulgare* and enzyme of cellulase from *Humicola insolens*.



α-amylase cellulase

Figure 1: α-amylase from *Hordeum vulgare* and cellulase from *Humicola insolens* [8].

3 Results and discussion

3.1 Protein concentration and nucleic acid 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 *H. werneckii* was centrifuged at 10000 rpm. The supernatant was measured by the UV-Vis spectrophotometer at 260 nm for the nucleic acid. The protein concentration in suspension of the cells was assayed by the Bradford method [9].

The protein concentration of the cell suspension *H. werneckii* after incubation in SC CO_2 was increased with increasing pressure and incubation time. *H. werneckii* contains many extracellular and intracellular proteins. Extracellular proteins are those enzymes which are completely separated from the cells and dissolved in the medium. Intracellular proteins were extracted from cells *H. werneckii* with incubation in SC CO_2 and consequently, the total protein concentration in the suspension of culture *H. werneckii* was increased. The highest

protein concentration in suspension of *H. werneckii* was found after 24 h of incubation in SC CO_2 at 300 bar (Figure 2).

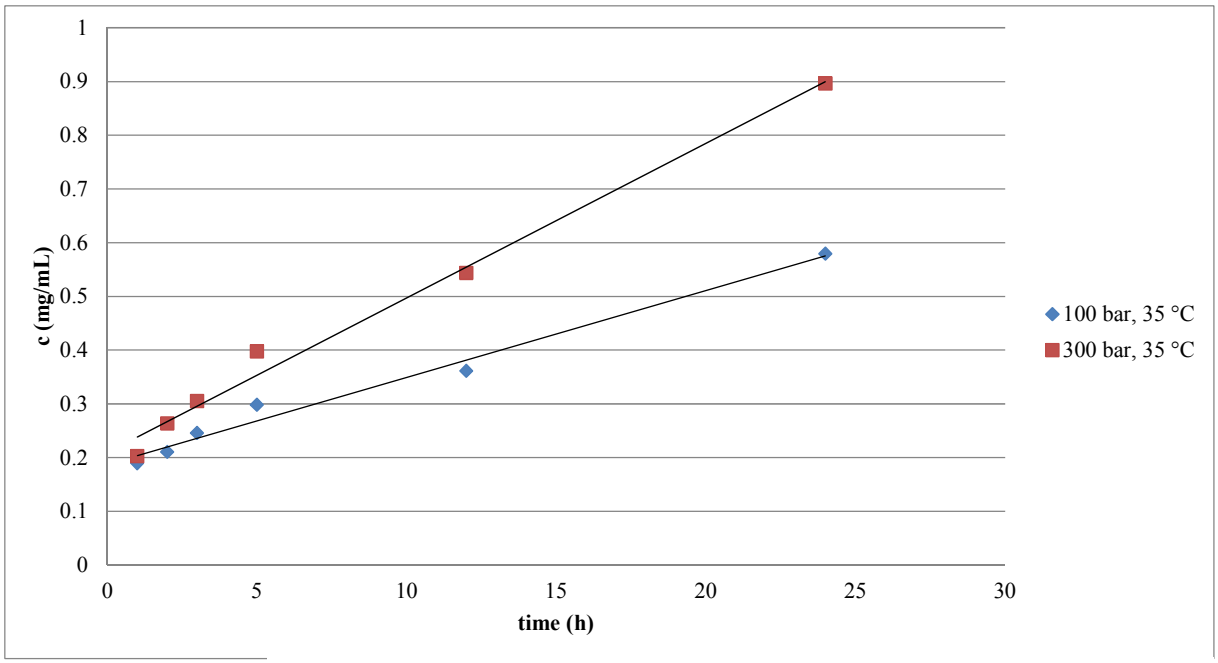


Figure 2: Protein concentration in *H. werneckii* suspension after incubation in SC CO_2 at different pressures (100 and 300 bar) and 35 °C.

Nucleic acids are also the most important protein macromolecules, which are found in abundance in all living cells. They are necessary for transmission of hereditary information from parent cell into daughter cells. With increasing the incubation time the absorbance of nucleic acids remained the same. Figure 3 shows that the absorbance of nucleic acids in the cell suspension, incubated at 300 bar, was higher than in the suspension, which was incubated at 100 bar. This was expected because at higher pressure more cells could be opened and more cellular material could be released.

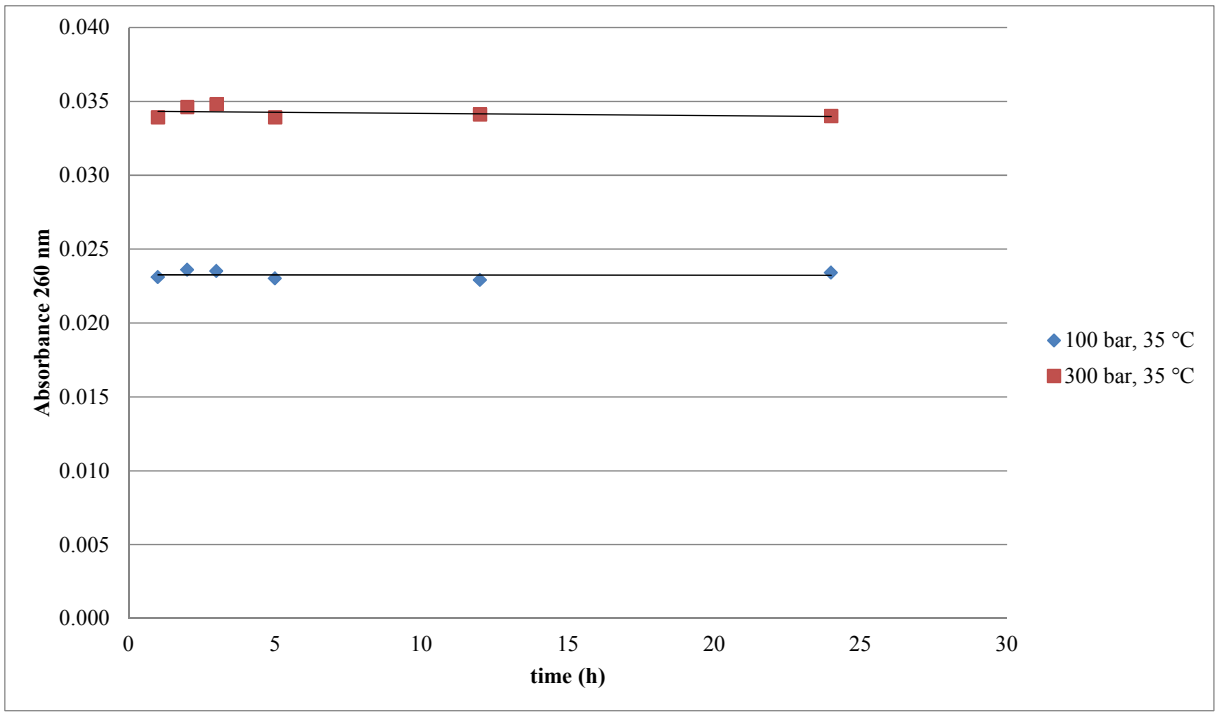


Figure 3: Absorbance of nucleic acid as a function of exposure time in SC CO_2.

3.2 Activity of cellulase and α-amylase

The activity of enzymes exposed to carbon dioxide under high-pressure depend on enzyme species, water content in the solution and the pressure and temperature of the reaction system. The three-dimensional structure of enzyme may be significantly altered under extreme conditions, causing their denaturation and consequent loss activity [1]. 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 protein which activity was determined in cell suspension of *H. werneckii* before incubation in SC CO_2. After exposure of cell suspension in SC CO_2 (Figure 4), the cellulase activity remained the same or started to decrease as a function of incubation time. Figure 4 shows the cellulase activity after exposure of cell suspension of *H. werneckii* in SC CO_2. The highest activity of cellulase after incubation of cell suspension *H. werneckii* in SC CO_2 was measured for 1 h at 100 bar. Cellulase activity was 15.63 unit/mL of cell suspension. With the increase of incubation time, the activity of cellulase in cell suspension *H. werneckii* was decreased, because the enzyme started to deactivate at both examined pressures (100 and 300 bar).

Amylase is in contrast to cellulase an intracellular enzyme. 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 amylase activity was increased. The cells of *H. werneckii* in suspension were opened and the enzyme was eliminated from cells in the medium. The highest activity of amylase was achieved at 1h and 300 bar (Figure 5). The amylase activity was 0.0095 unit/mL of cell suspension.

Higher pressure (300 bar) caused greater release of cells and consequently, as the result, more enzyme was released as in the case of incubation of cell suspensions in SC CO_2 at 100 bar.

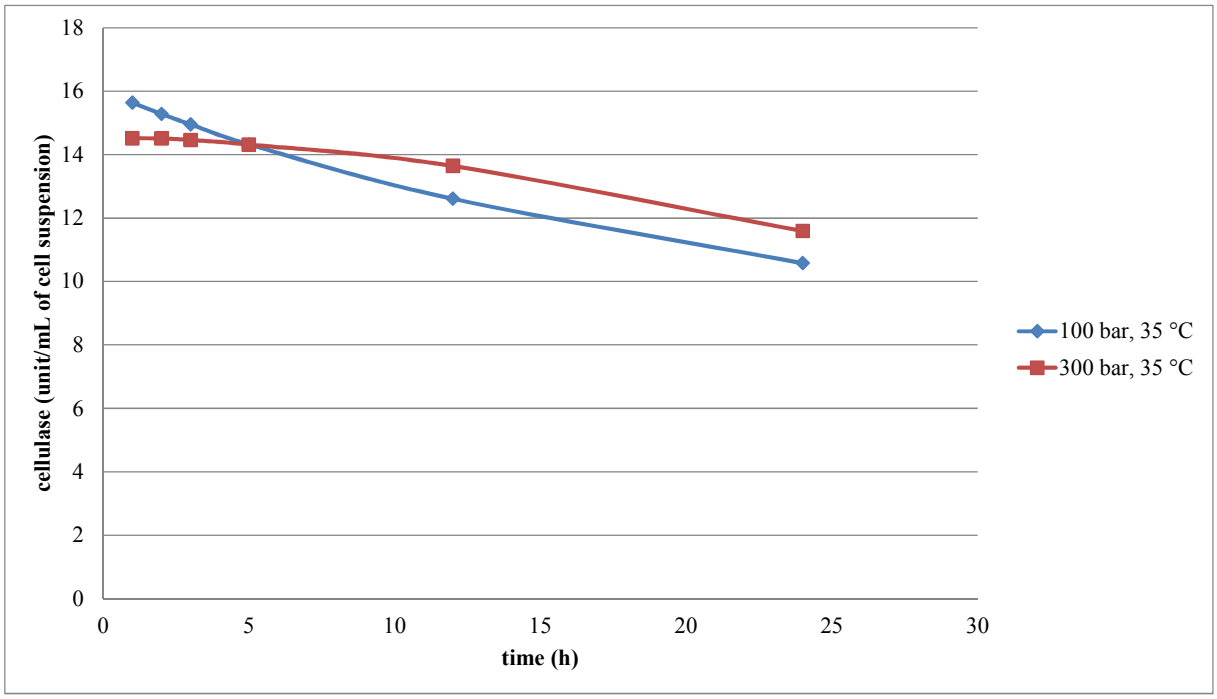


Figure 4: Activity of cellulase in *H. werneckii* suspension treated with SC CO_2 at 35 °C, 100 and 300 bar.

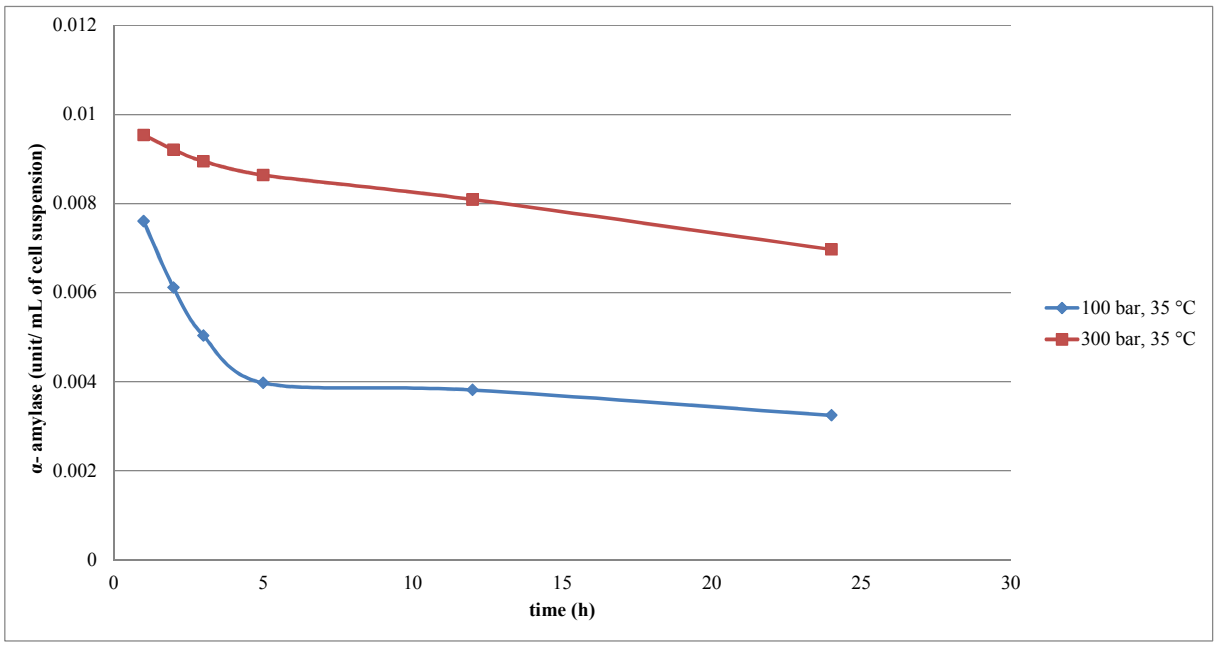


Figure 5: Activity of α-amylase in *H. werneckii* suspension treated with SC CO_2 at 35 °C, 100 and 300 bar.

3.3 Survival of *H. werneckii* by treatment of the SC CO_2

Black yeast *H. werneckii* is very resistant to the effects of high salinity, it can grow at very high salt concentration (32 %) [1]. Therefore our interest was to examine how is resists the increased pressure. Survival of *H. werneckii* cells in suspension under SC CO_2 was determined.

Survival of *H. werneckii* was monitored spectrophotometrically by measuring optical density of the cultures at 600 nm.
Figure 6 shows that after one hour incubation of cell suspension in SC CO_2 at 100 and 300 bar the survival of cells significantly decreased and were only 14 % and 12 %, respectively.
After 24 h incubation of cell suspension in SC CO_2, almost all cells of *H. werneckii* were dead. *H. werneckii* is not as resistant to high pressure as to high salt concentration.

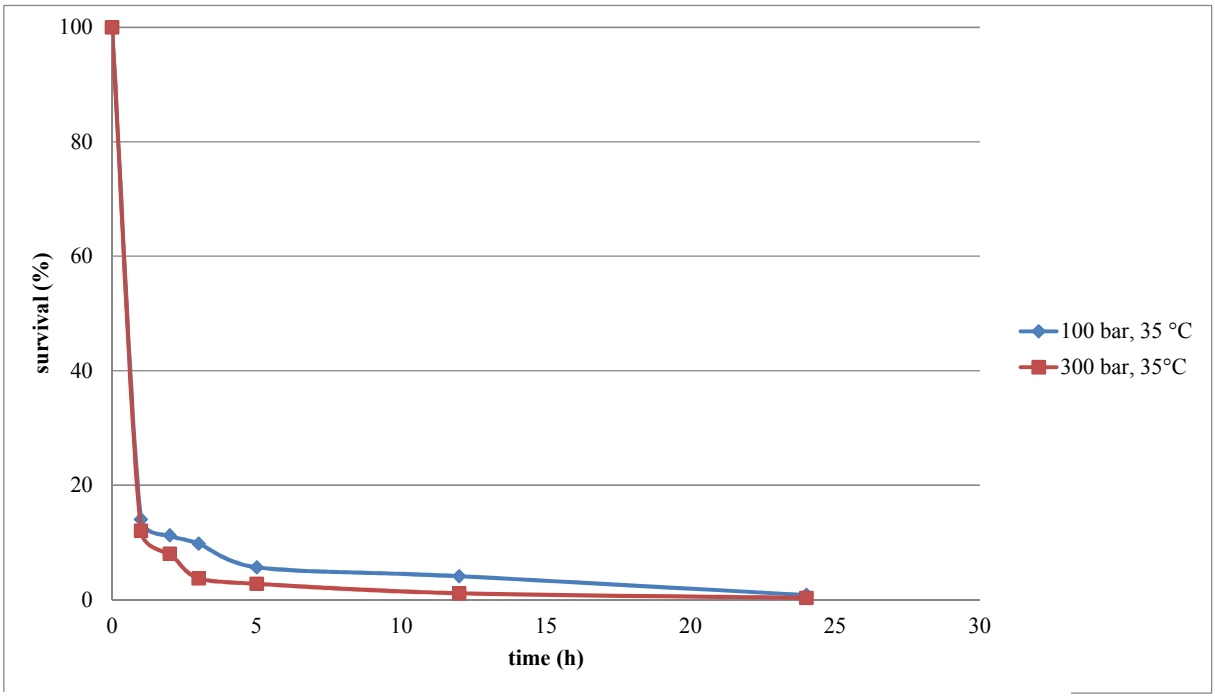


Figure 6: Effect of pressure and at 35 °C on the SC CO_2 treatment of *H. werneckii*.

4 Conclusion

The activity of enzymes from *H. werneckii* after incubation in SC CO_2 was studied. It was found that the cells of *H. werneckii* contain cellulase (extracellular enzyme) and amylase (intracellular enzyme). The highest activity of cellulase after incubation of cell suspension *H. werneckii* in SC CO_2 was achieved at 35 °C, 100 bar for 1 h and for amylase at 35 °C, 300 bar for 1 h. A large amount of proteins were extracted from *H. werneckii* cells. *H. werneckii* cells in suspension have reached almost complete inactivation after incubation for 24 h in SC CO_2 at both pressures.

5 References

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