P-074 **Immobilized cells of filamentous fungus *Aspergillus terreus* producing cellulases**

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INTRODUCTION

Cellulose present in plant biomass is a rich source of renewable raw material which can be converted into glucose under action of cellulases (50-65°C, pH 4.5-5.2). Glucose can be used for ethanol production as biofuel. It was shown, that usage of complex with cellulases possessing different hydrolytic activity is the most effective way, providing deep destruction of raw material components with high yield of monosaccharides. Filamentous fungi are the main microorganisms which produce cellulases (Saxena 2003).
Usage of immobilized cells of filamentous fungi, synthesizing enzymes, can create new approaches to production of effective biocatalysts for hydrolysis of cellulose-containing raw materials. Moreover, cell immobilization allows notable simplification of the production and application of biocatalysts.
Today, only a few works are known where immobilized filamentous fungi, synthesizing complex of cellulases, were investigated (Varfolomeev 2010, Hui 2010, Villena 2006, Haapala 1995, McCabe 2003, Lusta 2000). Some disadvantages of developed systems did not enable their use in practice.
The creation of immobilized biocatalysts (IBC), to obtain complex of cellulases with different substrate specificity, providing efficient hydrolysis of cellulose-containing substrates, was the main goal of present work. Poly(vinyl alcohol) cryogel (PVA CG) was used as the main carrier because of its chemical and mechanical stability as well as macroporous structure guarantying good conditions for mass-transfer processes (Lozinsky 1998).

MATERIALS AND METHODS

Immobilized cells of filamentous fungi *Aspergillus niger, A. terreus, Trihoderma harzianum, T. atroviride, Fusarium oxysporum, F. solani, Mucor circinelloides* were used as producers of cellulases. All microorganisms were obtained from VKM and VKPM. The following medium was used for microorganisms (g/l): glucose – 20, $MgSO_4$ – 0.2, $CaCO_3$ – 0.2, potato broth – 200, agar – 20 (pH 6.8).
The spore-containing suspension with concentration $4 \cdot 10^6$ spore/ml was mixed with 10% PVA solution to get homogeneous mass. It was frozen at -22°C. After 22 h mass was slowly defrosted at +8°C, cut into granules and placed for 100 h in following nutrient medium (g/l): $(NH_4)_2SO_4$ – 3.0, $MgSO_4 \cdot 7H_2O$ – 0.25, $ZnSO_4 \cdot 7H_2O$ – 0.25, K_2HPO_4 – 3.0, yeast extract – 5.0, glucose – 100 g, pH 5.5. Immobilized mycelium was grown up in this medium at 30°C and 180 rpm (Ellouz Chaabounis 1995).
The IBC (2 g in 100 ml) were cultivated in the same nutrient medium, containing 1-5% saw dust or rice stover instead of glucose at 28°C and 140 rpm.
The concentration of immobilized cells was controlled by bioluminescent method of determination of intracellular ATP concentration (Efremenko 2006).
Endo- and exo-gluconase and also β-glucosidase activities in culture medium were determined as it was described previously (Hui 2010). Enzymatic activity (U) was expressed either as μmol glucose (for β-glucosidase) or glucose equivalent (for endo- and exo-gluconase) liberated per min.

RESULTS AND DISCUSSION

The screening of most effective producer of cellulases between different filamentous fungi biosynthesizing extracellular enzymes was carried out using soluble (carboxymethilcellulose (CMC)) and insoluble (Avicel) substrates. Maximal endo- and exo-gluconase and also β-glucosidase activities were obtained at different phases of cultivation of filamentous fungi *Aspergilus terreus*. This strain was taken for immobilization into PVA CG.
Immobilized spores of fungi were grown up for 100 h at 30°C and 180 rpm in the nutrient medium which was described above for obtaining of IBC with activated metabolism. It was shown that intracellular ATP concentration in granules of IBC increased in 10 times as compared to its initial level corresponding to IBC formation (from 1.8×10^{-7} to 6.4×10^{-6} mol/g_{IBC}). It indicated good physiological status of immobilized cells of filamentous fungi.
Formed IBC was placed into minimal medium containing saw dust as sole carbon source and inductor of biosynthesis of cellulolytic complex. Also the secretory function of IBC taken without preliminary formation was studied. It was shown that secretion of cellulases by IBC, taken without preliminary formation on the medium containing 10% glucose, was essentially higher than same characteristic of formed IBC. Maximal endo-gluconase and β-glucosidase activities (1760 u/l and 147 u/l, respectively) were obtained in the cultural medium with immobilized cells of filamentous fungi *A. terreus* after 15 days of cultivation. Maximal exo-gluconase activity (89 u/l) was obtained in the medium after 7 days.
Maximal endo- and exo-gluconase and also β-glucosidase activities (4360 u/l, 128 u/l and 280 u/l, respectively) of cellulolytic complex secreted by developed IBC were obtained in the media containing 5% of rice stover as sole

carbon source after 7 days. It was demonstrated that biosynthetic function of this IBC hold at the same level for at least 5 cultivation cycles with replacement of medium after each cycle (Fig. 1). It demonstrated high enough operational stability of developed IBC.

Corresponding to presented results, the developed IBC provided 9.7 units of exo-gluconase activity at day per liter of cultivation medium, which was 30% higher than productivity of IBC previously described in literature (Hui 2010). It is necessary to note, that results of this work were obtained using natural substrate (rice stover) oppositely to commercially available lightly fermentable pure cellulose used by other authors in similar investigations (Hui 2010, Lusta 2000).

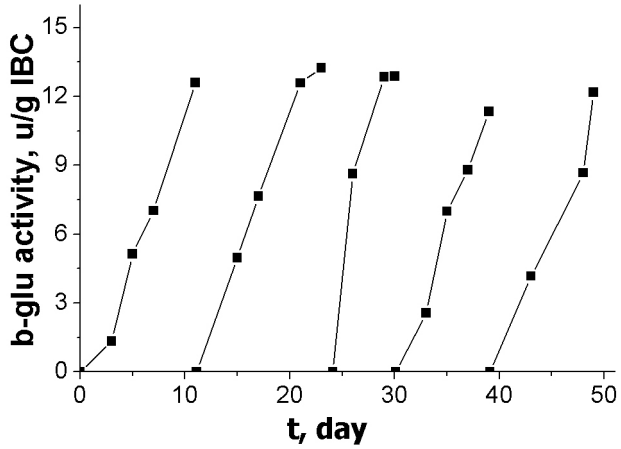

Fig. 1. β-glucosidase activity, secreted by immobilized filamentous fungi *Aspergillus terreus* in medium under fed-batch cultivation conditions for 5 cycles with duration of each cycle from 7 to 10 days.

It was revealed, that cultural medium obtained after IBC cultivation could lose up to 80% of endo-gluconase and β-glucosidase activities during storage for 60 days at +8°C. The inactivation constants of enzymes of cellulolytic complex under these conditions were equal to $(4.53\pm0.23)\cdot10^{-8}$, $4.53\pm0.23)\cdot10^{-8}$, $4.53\pm0.23)\cdot10^{-8}$ for endo-, exo-gluconase and β-glucosidase, respectively (Fig. 2).

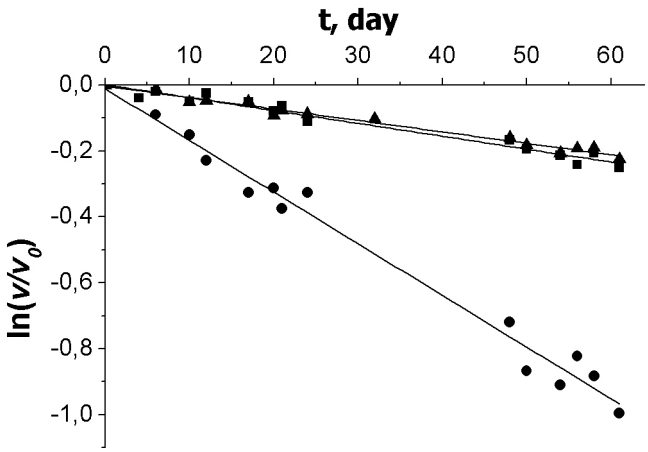

Fig. 2. Inactivation of endo-, exo-gluconase and β-glucosidase in medium obtained after immobilized cells *Aspergillus terreus* during its stored at +8°C.

Active cultural medium was used to hydrolyze rice stover after preliminary delignification. It was shown that glucose appeared in the medium after 24 h incubation at 42°C as result of hydrolytic action of present cellulases.

CONCLUSION

Possible development of IBC based on filamentous fungi producing cellulases and PVA CG was shown for the first time. Developed IBC enabled synthesis of cellulolytic complex in minimal medium, containing natural substrate as sole carbon source and inductor of biosynthesis. Enzymes accumulated in medium could be used for effective hydrolysis of cellulose-containing raw materials.

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