Encapsulated in sol-gel biohybrids *Humicola lutea* cells cultivated in bioreactor for repeated batch α-galactosidase synthesis

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INTRODUCTION

The enzyme α-galactosidase (EC 3.2.1.22), catalyzing the hydrolysis of α-1, 6-linked galactosyl residues in oligosaccharides and galactomannans has a wide application in biotechnology for the nutritional improvement of legumes based foods and fodders (de Resende et al. 2005). It is known that free cells of the mesophilic filamentous fungus *Humicola lutea* 120-5 produce high level of α-galactosidase (Aleksieva et al. 2007). Since now no data exists about the immobilization of microorganisms including filamentous fungi, producers of α-galactosidase. The published repots concern only the immobilization of this enzyme from fungi in different carriers Ca alginate (Prashanth and Mulimani 2005), chitosan (Kulkarni et al. 2006), polyacrylamide (Thippeswamy and Mulimani 2002), carrageenan (Girigowda and Mulimani 2006).
Sol-gel processes give new possibilities in the field of chemistry and science. Using the sol-gel method new materials can be synthesized. Hybrid materials formed by the nanoscale inorganic and organic domains are attractive for the purpose of creating new materials compared to the organic or inorganic materials separately (Sgarbi et al. 2004). The hybrid is formed in situ in a biopolymer solution by self-assembling of sol particles generated in the course of hydrolysis of the metal-organic precursors (Hamano et al. 2004; Cho et al. 2004; Gill et al. 2000).
In our previous works we have established an enhancement of acid proteinase production by this strain immobilized in crosslinked poly(vinyl alcohol) mixed with poly(ethylene glycol), (Aleksieva et al. 1998). The silica hybrids containing polyethylene glycol, polyvinyl alcohol and other organics have been synthesized by the sol-gel method (Samuneva et al. 2002; Chernev et al. 2005).
In this study we report on α-galactosidase production in airlift bioreactor, using *Humicola lutea* cells immobilized in sol-gel matrix consists of tetraethylortosilicate (TEOS) and algal polysaccharide (AlPS). The growth tendency of the immobilized mycelium and its distribution on the carrier surface was observed with scanning electron microscopy.

MATERIALS AND METHODS

The fungal strain *Humicola lutea* 120-5 (National Bank for Industrial Microorganisms and Cells Cultures, Bulgaria, № 391) was used in this investigation.
The hybrid sol-gel matrix was prepared at room temperature as films using as a silicon precursor tetraethylortosilicate (TEOS) purchased by "Merck" and different quantities of Dixonella's polysaccharide (20 and 40 wt %). A poly-step sol-gel procedure is applied at strictly controlled conditions in order to obtain the desired nanostructured materials. In all cases the ratio precursor / H_2O / 0.1 N HCl is kept 1 / 1 / 0.01. The acid is introduced to increase hydrolysis rate (pH ~ 1.5). Cell immobilization was carried out using spore suspension (5 ml) with a density 10^{10} spores per ml from one test tube. The sol-gel particles containing the entrapped spores obtained after 72 h of drying of the film as well as the free spores were used as an inoculum in the batch α-galactosidase biosynthesis in an airlift bioreactor.

α-Galactosidase activity was assayed [18-20] using 0.003 M *p*-nitrophenyl-α-D-galactopyranoside as substrate in a citrate phosphate buffer, pH 5.5. One unit (U) of α-galactosidase activity was defined as the amount of enzyme which liberates 1 μmol of *p*-nitrophenol per min under the described conditions.

RESULTS AND DISCUSSION

For studying the structure of synthesized hybrids the following methods have been used: FT-IR (IR- MATSON 7000–FTIR spectrometer), XRD (X-ray PW1730/10 diffractometer), BET-Analysis (Gemini 2370 V5.00), SEM (Philips-515) and AFM (NanoScope Tapping ModeTM).
The results from the XRD-analysis show that all the studied hybrids are in amorphous state (Fig. 1). It is visible that with introducing of organic component into synthesized nanobiomaterials, intensity of peaks decreases.
The FT-IR spectra of synthesized inorganic-organic materials have shown that in all samples bands at 1080 cm^{-1}, 790 cm^{-1} and 480 cm^{-1} are observed. They are assigned to vas, vs and δ of Si-O-Si vibrations, but at the same time the band at 1080 cm^{-1} can be related to the presence of Si-O-C, C-O-C and Si-C bonds. The band at 960 cm^{-1} is due to a stretching Si-OH vibration. The band at 1439 cm^{-1} is assigned to C-O-H vibrations. The characteristic bands at around 3450 cm^{-1} and at 1620 cm^{-1} assigned to H-O-H vibration can also be detected.
The presence of a hybrid nanostructure with well-defined nanounits and their aggregates, formed by self-organizing processes, is clearly observed by AFM studies. The size of nanoparticles is from 3 to 7 nm (Fig. 3, 4). Figure 3b, 4b shows the height distribution profiles of surfaces roughness. The histograms of the surface height distribution profiles, obtained from AFM images, show that the inorganic-organic hybrid sample has surface with irregularities of quite small height. The surface morphology and structure of nanobuilding blocks in each synthesized hybrid is different and depends on its chemical composition. In the sample the nanoparticles are well distributed in the entire hybrid matrix with a lower degree of aggregation, while the sample prepared with TEOS showed that the nanoparticles are self-organized and distributed as clusters in the matrix. Although all being amorphous, quite different self-organized structures can be observed in these hybrids.

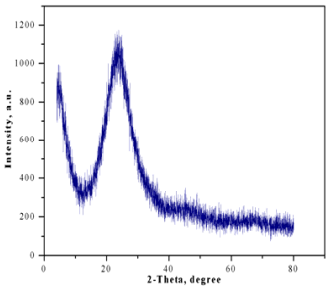

Fig. 1. XRD patterns of silica hybrids containing TEOS and 20 wt. % AIPS

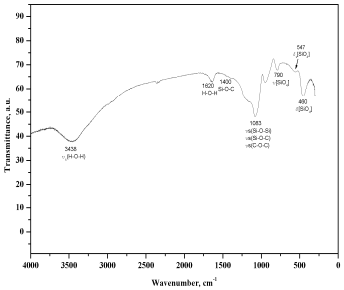

Fig. 2. FT-IR spectra of silica hybrids containing TEOS and 20 wt. % AIPS

The influence of the concentration of organic part (AlPS) in the hybrid matrix on the α-galactosidase of immobilized mycelium as compared to the free cells during bioreactor cultivation is presented in Fig. 5. As can be seen free biomass produced higher α-galactosidase activity, reaching maximal level (750 U/l or 100%) at the 120th h. In the case of immobilized *H. lutea* cells

the maximal values of the enzyme titre reached at the 144th h were 410 U/l and 560 U/l for the samples with 20 and 40% AlPS, respectively. These lower results could be explained by the barrier formed by the carrier to the nutrient supply for mycelia formation during the precultivation of entrapped spores (growth phase), as it was previously discussed.

Fig. 6 demonstrates the growth of *H. lutea* hyphae obtained after precultivation of free and entrapped spores in hybrid matrices containing 20 and 40% AlPS.

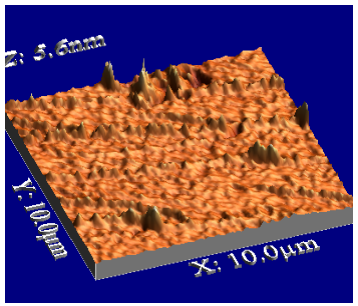

Fig. 3. AFM image and height distribution profile of surface roughness of hybrid material containing 20 wt. % AIPS.

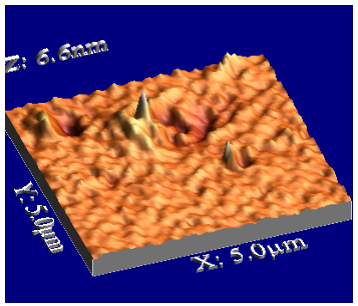

Fig. 4. AFM image and height distribution profile of surface roughness of hybrid material containing 40 wt. % AIPS.

Repeated batch bioreactor cultivation of immobilized H. lutea mycelium

144-old immobilized mycelium obtained after precultivation of spores entrapped in sol-gel matrix containing 40% AlPS was used as an inoculum per nutrient medium in the first batch. Fig. 7 demonstrates α-galactosidase yield of immobilized *H. lutea* cells during repeated batch cultivation in an airlift bioreactor. The maximal enzyme level, reaching in the fifth cycle was 1200 U/l or 160% as compared to the free cell fermentation (750 U/l).

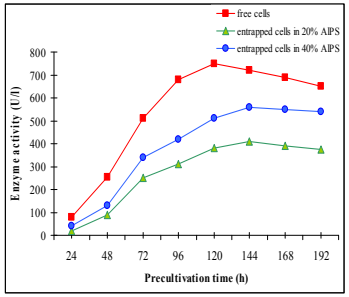

Fig. 5. Influence of the concentration of organic part in the hybrids on the α-galactosidase activity during precultivation.

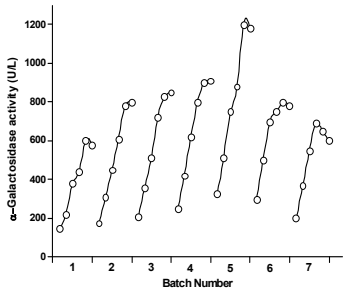

Fig. 6. Repeated batch bioreactor cultivation of *H. lutea* mycelium immobilized in hybrids.

The advantages of this biocatalyst are: (i) the half-life time of enzyme production was 42 days (7 batches), whereas in the shake-flask experiments it is only 3 cycles (or 18 days) with maximal

enzyme titre which does not exceed the activity of free cells. (ii) During a long period of a month (from second up to sixth cycle) the level of α-galactosidase exceeded the free mycelium fermentation.

CONCLUSIONS

Finally we can say that the synthesized matrix on the basis of TEOS and algal polysaccharide was successfully used in the immobilization of *H. lutea* cells. They are entrapped in the hybrid matrix and continue their development as a mycelium which attaches to the surface, while the biosynthetic capability is preserved and the values for the enzyme titer exceeded three-fold the α-galactosidase activity of the free cells. In conclusion, the high operational stability of TEOS - AlPS (40%) biocatalyst as well as the long half-life time of α-galactosidase production can facilitate continuous process in an airlift bioreactor.

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