Hybrid sol-gel matrices for *H. lutea* immobilization for semicontinuous α-galactosidase production

G. Chernev[1], P. Aleksieva[2], L. Nacheva[2], L. Kabaivanova[2], B. Samuneva[1], P. Djambaski[1], I. M. Miranda Salvado[3]
[1]University of Chemical Technology and Metallurgy, Sofia, Bulgaria
[2]Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria
[3] University of Aveiro, CICECO, Portugal
georgi_chernev@yahoo.com

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 the present study spores of *Humicola lutea*, as well as 24-h mycelium were entrapped in the hybrid sol-gel matrix, containing TEOS or MTES as precursors and a mixture of PVA and PEG. The incorporated spores and vegetative cells were precultivated for 120 h for fungal development in the matrix. The obtained immobilized mycelium was employed for shake-flask semicontinuous α-galactosidase production.

Materials and Methods

For hybrid materials synthesis different types of inorganic silica precursors and organic materials have been used. The main inorganic precursors are tetraethylortosilicate (TEOS) and methyltrietoxysilane (MTES). 0.1 N HCl and phosphate buffer with pH=7.00±0.02 at 20^{o}C were also used in the investigations. A small amount of 0.1 N HCl is introduced in order to increase hydrolysis rate (pH~1, 5). The inorganic-organic hybrid matrices have been prepared by substituting part of the inorganic precursor with PEG and PVA as organic constituents. Thus synthesized matrices were applied as carriers for cell immobilization. The fungal strain *Humicola lutea* 120-5, registered in the National bank for industrial microorganisms and cell cultures: 391, Sofia, Bulgaria (Grigorov et al. 1983) was used in this study.
As a nutrient medium soy meal waste extract (5% dry content) was used, according to Aleksieva et al., (2007). The washed and dried sol-gel particles with the entrapped spores or vegetative cells were cultivated in 500 ml Erlenmayer flasks with 50 ml mediun in a rotary shaker (220 rpm) at

30°C. After 120 h the particles with the immobilized mycelium were transferred into fresh medium at 144-h and 216–h intervals during the repeated batch use.
Fungal growth and distribution of the hyphae in the sol-gel matrix were observed using Light microscopy (Carl Zeizz, Yena). Transmission electron microscopic observations were made on EM 10C. Spore suspension (6 ml, density 10^{10} spores/ml) and 24-h mycelium (dry weight 0.23 g/flask) were used as biomaterial for entrapment in the hybrid sol-gel matrix. α-Galactosidase activity was assayed by the method of Dey et al., (1993).

Results and Discussion

The results from the XRD - analysis prove that all the studied hybrids have an amorphous structure. At the same time the sharpening of the amorphous halo indicates that some processes of ordering are carried out.
The FT-IR spectra of synthesized inorganic-organic materials show that in all samples bands at 1080 cm^{-1}, 790 cm^{-1} and 480 cm^{-1} are observed. They are assigned to ν_{as}, ν_s and δ of Si-O-Si vibrations, but at the same time these bands 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. These bonds in the samples with MTES are in a narrower range compared to IR spectra of samples with TEOS. The absorption bands at 2975 cm^{-1}, 1255 cm^{-1}, 880 cm^{-1} and 694 cm^{-1}, due to the presence of Si-O-R (CH_3 and C_2H_5) and Si-C bonds have been observed only in the samples containing MTES. This fact directly proves the presence of strong chemical bonds between inorganic and organic parts of synthesized materials.

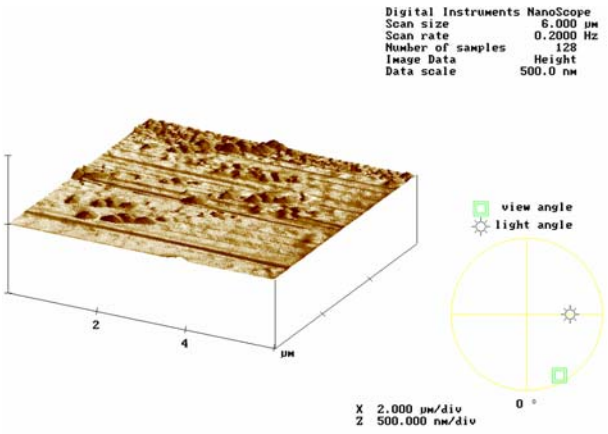

Fig. 1. AFM images of the hybrids (TEOS) containing PEG+PVA.

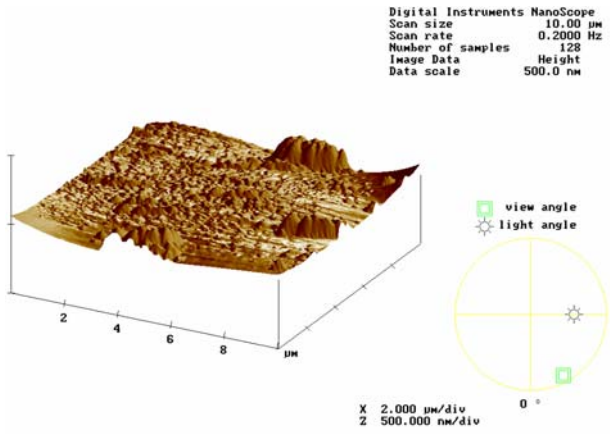

Fig. 2. AFM images of the hybrids (MTES) containing PEG+PVA.

From the data of BET analysis it has been established that the pore size is in the range of 1 to 1,8 nm. With increasing the percent of organic part, the pore size decreases. The presence of a heterogeneous structure with well-defined nano units is clearly seen from the AFM studies (Fig. 1, 2). The average size of nanoparticles on the sample surface is about 30 nanometers and the formation of their self-organized structures can be observed.
The synthesized hybrid matrices were used for the encapsulation of spores and vegetative cells. After precultivation of entrapped spores and 24-h mycelium, as well as free spores for 120 h the hyphae formation was followed. The Light microscopy picture of the free mycelia shows elongated hyphae in a network (Fig. 3). After 120 h a fragmentation of the filaments and spore formation was established. Fungal development in the whole interior of the sol-gel particle was observed (Fig. 4).

As a result of drying when using young mycelium (24 h), some damages like folding and tearing and decreased turgor were registered. These changes in the mycelial growth were probably due to the three day period of drying of the matrix with the included vegetative cells.

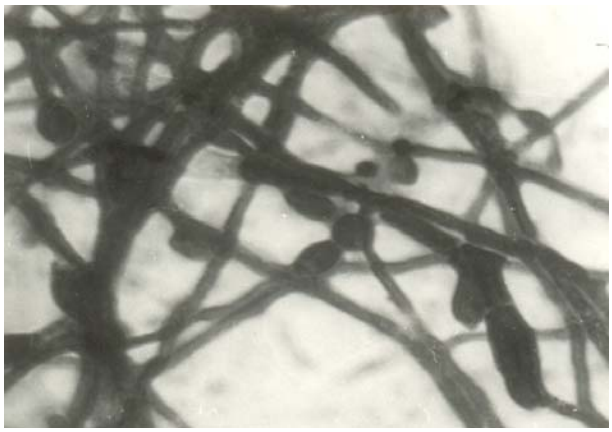

Fig. 3. Light microscopy of free mycelia network composed from elongated thick hyphae and spore formation after 120 h submerged cultivation. x 2500

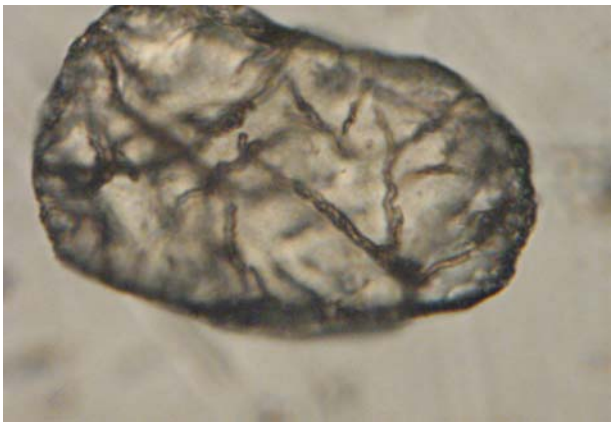

Fig. 4. Light microscopy of immobilized mycelia in a piece of sol-gel matrix after 120-h of precultivation of entrapped spores. x 160

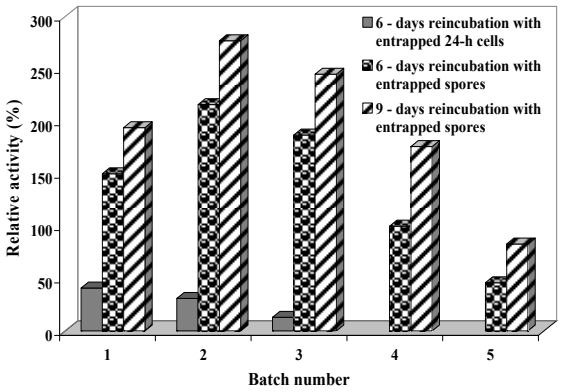

Fig. 5. Semicontinuous α-galactosidase production by immobilized mycelium.

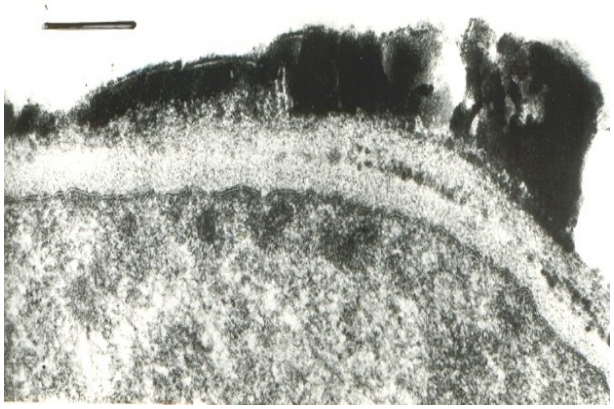

Fig. 6. TEM of an ultrathin section of a *H. lutea* 120-5 spore. (Bar=0, 2 µm)

The sol-gel pieces, containing immobilized mycelium originating from spores and vegetative cells were used in a repeated batch mode. Figure 5 demonstrates the semicontinuous α-galactosidase production with different duration of the reincubation periods. The vegetative cells appeared to be not suitable biomaterial for incorporation in this matrix because the maximal activity (first cycle) is only 41% from the control level. The control (100%) represents α-galactosidase activity (24 U/flask) obtained for 144 h or 6 days fermentation of free cells. At entrapment of spores and 6 days reincubation the maximal activity exceeds two-fold (216%) the activity of the control. Half–life time of enzyme production was 4 cycles (24 days). At the same conditions, but 9 days reincubation period, the maximal yield compared to free cells reached 60 U/flask or 277% and half life time was also 4 cycles, but 36 days. Similar results concerning acid proteinase production by *H. lutea* immobilized in crosslinked PVA mixed with PEG were previously obtained (Aleksieva et al. 1998). The better results for the entrapment of spores can be due to the presence of an electron dense zone

with a melanin layer covering the spore wall (Fig. 6). The investigations on the melanin function prove that its presence in the spore wall is connected to the survival of the microorganism under unfavorable conditions. The melanin acts as a barrier that hinders from drying (Bertoldi et al. 1974).

Conclusions

On the basis of the obtained results we can conclude that the synthesized hybrid materials on the basis of TEOS or MTES and mixture of PEG and PVA with their characteristic features allow the successful immobilization of the mycelium of *H. lutea* by incorporation of spores capable of α-galactosidase synthesis.

Acknowledgements

This work was supported by Grant NT2-01 and NT2-02 of the National Fund for Scientific Research, Republic of Bulgaria.

Bibliography

1. Aleksieva P et al. (1998) *Enhancement of acid proteinase production by fungus Humicola lutea 120-5 immobilized in crosslinked poly(vinylalcohol) mixed with poly(ethylene glycol). Process Biochem*., 33(7), 725-728.
2. Aleksieva P et al. (2007) *Production of extracellular α-galactosidase by mesophilic filamentous fungi during submerged cultivation. Compt. Rend. Acad. Bulg. Sci*., 60(6), 691-696.
3. Bertoldi M et al. (1974) *The fine structure of some strains of Humicola traaen.* Can. J. Microbiol, 54, 2755-2768.
4. Dey P et al. (1993) *Induction of alpha-galactosidase in Penicillium ochrochloron by guar (Cyamopsis tetragonobola) gum.* Biotechnol. Appl. Biochem., 17, 361-371.
5. Girigowda K et al. (2006) *Hydrolysis of galacto-oligosaccharides in soymilk by k-carrageenan-entrapped α-galactosidase from Aspergillus oryzae.* World J. Microbio. Biotechnol., 22, 437-442.
6. Grigorov I et al. (1983) *Selection of gamma-ray mutants from a strain of Humicola lutea 72 producing acid proteases*. Eur. J. Appl. Microbiol. Biotechnol., 17, 355-357.
7. Kulkarni D et al. (2006) *Reduction of flatus-inducing factors in soymilk by immobilized alpha-galactosidase.* Biotechnol. Appl. Biochem., 45, 51-57.
8. Prashanth S et al. (2005) *Soymilk oligosaccharide hydrolysis by Aspergillus oryzae α-galactosidase immobilized in calcium alginate.* Process Biochem., 40, 1199-1205.
9. de Rezende S et al. (2005) *Purification and characterization of an α-galactosidase from Aspergillus fumigatus.* Braz. Arch. Biol. Technol. 48, 195-202.
10. Thippeswamy S et al. (2002) *Enzymic degradation of raffinose family oligosaccharides in soymilk by immobilized α-galactosidase from Gibberella fujikuroi.* Process Biochem., 38, 635-640.
11. Hamano Y *et. al.* (2004) *Preparation of flexible inorganic-organic hybrid proton-conducting membranes via sol-gel processing*. Journal of Materials Science 39, 7097-7099
912 Sgarbi N *et. al.* (2004) *Self-assembled extracellular matrix protein networks by microcontact printing*. Biomaterials 25, 1349-1353
13. Cho J *et. al.* (2004) *Influence of silica on shape memory effect and mechanical properties of polyurethane–silica hybrids.* European Polymer Journal 40, 1343-1348.
14. Gill I *et al.* (2000) *Bioencapsulation within synthetic polymers (Part 2): non-sol–gel protein–polymer biocomposites*. Tibtech 18, 469-480
15. Chernev G *et. al.* (2005) *Silica nanocomposites containing polyacrilamide gel*. Glass Sci. & Tech. 179-184.
16. Samuneva B et al (2002) *Sol – gel syhthesis of glassy hybrid materials for cell immobilization, Glass Science and Technology* 75 C2, 434-437.