# Examination of *Humicola lutea* Immobilized in Sol-Gel Matrices: Effective Source of α-Galactosidase

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α-Galactosidase production by the fungus *Humicola lutea* 120–5 immobilized in a hybrid sol-gel matrix, consisting of tetraethylorthosilicate (TEOS) as a precursor and a mixture of polyethyleneglycol (PEG) and polyvinylalcohol (PVA), was investigated under semicontinuous shake flask cultivation and compared to the enzyme secretion by free cells. The influence of the carrier weight on the α-galactosidase biosynthesis in repeated batch experiments was followed. Best results were obtained with 2 g of the sol-gel particles per culture flask using 144-h runs. The growth behaviour of the immobilized mycelium during both the growth and productive phases was observed by scanning electron microscopy. The presence of abundant mycelial growth of intact hyphae correlated with a 2-fold higher enzyme activity compared to free cells. The obtained biocatalyst retained a high level of enzyme titer exceeding the activity of free cells during four cycles of operation (24 days). This result is confirmed by the micrographs showing the retained viability of the growing vegetative cells due to the protective role of the carrier.

*Key words:* Immobilization, Fungi, Scanning Electron Microscopy

## Introduction

α-Galactosidase (EC 3.2.1.22) catalyzes the hydrolysis of the terminal non-reducing α-galactosyl residues from oligosaccharides, galacto(gluco)-mannans and galactolipids (Margolles-Clark *et al*., 1996). This enzyme can be used in the production of sugar, in the processing of soymilk for removing antinutrient oligosaccharides, in the conversion of blood type and in the treatment of the Fabry disease (Ganter *et al*., 1988; Prashanth and Mulimani, 2005; Lenny *et al*., 1994).

Fungal α-galactosidase attracts much attention in term of its suitable pH value, fine stability and extracellular secretion (Mi *et al*., 2007). In the past few years, many workers reported on the immobilization of fungal α-galactosidases in different carriers and their application in the removal of flatulence-inducing sugars in soymilk (Naganagouda *et al*., 2007; Girigowda and Mulimani, 2006; Prashanth and Mulimani, 2005).

To our knowledge, there are no published reports on the immobilization of fungal cells producing α-galactosidase. We firstly reported on the α-galactosidase production by immobilization of the fungus *Humicola lutea* 120–5 in sol-gel matrices (Chernev *et al*., 2007; Kabaivanova *et al*., 2006). In our previous works we have investigated the growth tendency of *H. lutea* mycelium immobilized in a crosslinked prepolymer for acid proteinase biosynthesis (Aleksieva *et al*., 1999) as well as the growth and distribution of the hyphae of the same fungal strain at immobilization in a polyurethane sponge for acid phosphatase production (Aleksieva *et al*., 2003). No data is available on the scanning electron microscopy (SEM) observations of the fungal development in a sol-gel hybrid matrix.

Here we describe the growth behaviour of *H. lutea* mycelium immobilized in a hybrid matrix and the investigation of the morphology using SEM. The matrix was synthesized by a sol-gel method and it consists of tetraethylorthosilicate (TEOS) as an inorganic precursor with the addition of polyethyleneglycol (PEG) mixed with polyvinylalcohol (PVA). The effect of the carrier weight on the α-galactosidase production during semicontinuons shake flask fermentation compared to the enzyme secretion by free cells was also studied.

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## Materials and Methods

### *Microorganism and medium*

The fungal strain *Humicola lutea* 120–5 (National Bank for Industrial Microorganisms and Cell Cultures, Bulgaria, No. 391) was used in the present study. The culture was maintained on 1.5 % (w/v) beer agar at 28 °C for 7 d to obtain dense sporulation. Soya meal extract (SME) (Aleksieva *et al.*, 2007) was used as a nutrient medium for growth as well as α-galactosidase production by both free and immobilized cells.

### *Immobilization procedure and culture conditions*

Sol-gel transparent silica hybrid matrices have been synthesized at room temperature under strictly controlled pH conditions. The silicon alcoxide precursor TEOS (Merck) has been used. In all cases the ratio precursor/$H_2O$ was kept constant and equal to 1. A small amount of 0.1 M HCl was introduced to increase the hydrolysis rate (pH ~1.5). Then a phosphate buffer [pH (7.0 ± 0.02)] was rapidly added to raise the pH value to 6.0, suitable for keeping the cell vitality. Different quantities of organic compounds: PEG ($M_r$ 400), PVA ($M_r$ 82000) were added to obtain the hybrid material. The gelation time was less than 1 min for all samples after cell addition and no phase separation was observed.

6 ml of the spore suspension ($10^{10}$ spores/ml) were entrapped in the matrix. The washed and dried sol-gel pieces with the entrapped spores and free spores were precultivated for 144 h in 500-ml Erlenmeyer flasks with 50 ml medium in a rotary shaker (220 rpm) at 30 °C for conidial fermentation and mycelium formation. Then the washed particles containing immobilized biomass as well as free mycelium were ready for use as an inoculum for semicontinuous α-galactosidase production.

### *Repetition of batch culture*

The effect of the carrier weight on the α-galactosidase production during repeated use in sequential batch cultures was investigated using two samples of immobilized mycelium prepared with 1.0 and 2.0 g sol-gel matrix per flask. Repeated batch experiments were carried out in duplicate under the conditions described above. At the end of each batch (144 h), both immobilized mycelium and free biomass (as control) were washed and transferred into fresh medium to start a new run. The culture filtrates were assayed for the α-galactosidase activity, expressed as units per flask. During the experiments with immobilized cells, the deviation of replicates was less than 3 %, whereas free cell fermentation demonstrated a deviation of 5–6 %.

### *Enzyme assay*

The α-galactosidase activity was assayed (Dey *et al.*, 1993) using 0.003 M *p*-nitrophenyl-α-D-galactopyranoside as substrate in a citrate-phosphate buffer, pH 5.5. The reaction mixture was incubated at 50 °C for 15 min; the reaction was stopped by the addition of 0.1 M sodium carbonate. The amount of released *p*-nitrophenol was measured by the molecular absorbance at 405 nm. 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.

### *Scanning electron microscopy (SEM)*

For the SEM observations the samples of *H. lutea* free cells and entrapped spores were fixed for 2 h with 2 % (w/v) glutaraldehyde (Angelova *et al.*, 2006) at room temperature. After washing with distilled water samples were dehydrated in ethanol series ranging from 30 to 100 % (v/v). After air-drying, specimens were coated with 120–130 Å gold in argon atmosphere using an Edwards apparatus (model S 150A). The SEM observations were made on a Philips SEM 515 at 20 kV accelerating voltage with a 5–6 nm electron beam.

## Results and Discussion

### *Influence of the carrier weight on α-galactosidase production during repeated batch shake flask cultivation*

Fig. 1 presents the α-galactosidase production (U/flask) by *H. lutea* mycelium immobilized in a sol-gel matrix with a weight of 1.0 and 2.0 g/flask as well as by free cells during repeated use in sequential 144-h batch cultures. The use of 2.0 g pieces containing immobilized mycelium resulted in higher enzyme yield. The α-galactosidase level rapidly increased during the first three reincubations reaching a maximal value of 52 U/flask in the second batch, which is about two-fold higher than the activity in the control sample (24 U/flask or

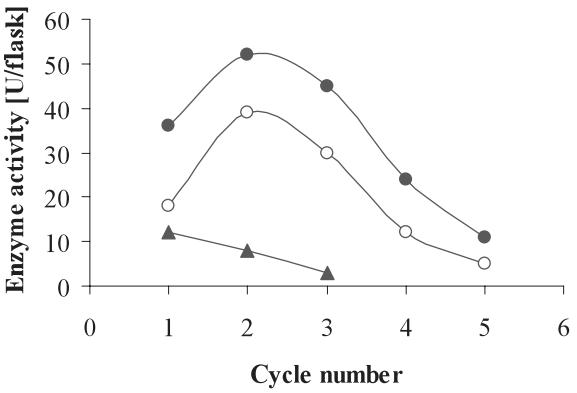


Fig. 1. α-Galactosidase production by *H. lutea* mycelium in a sol-gel matrix with a carrier weight of 1.0 g (○) and 2.0 g (●) as well as by free cells (▲) cultivated in 50 ml soya meal extract per flask as nutrient medium during repeated use in sequential 144-h batch cultures.

100 % obtained in 144 h of free cell fermentation). When the weight of the carrier was lower (1.0 g/flask) the α-galactosidase yields in the batches were smaller as compared to the experiments with higher carrier content. In this case the maximal enzyme level (39 U/flask or 162 %) was also reached in the second cycle. The increase of α-galactosidase production in the medium with a greater weight of the carrier at equal starting quantities of entrapped spores (6 ml, $10^{10}$ spores/ml) may result in an increase in the diffusion surface and consequently a better nutrient and oxygen supply as well as enzyme excretion throughout the matrix. Similar results were obtained by Aleksieva *et al.* (2003) investigating the effect of the polyurethane sponge weight on acid phosphatase production by immobilized *H. lutea*.

When free *H. lutea* spores were precultivated for 144 h for mycelium formation the α-galactosidase level reached 24 U/flask. In repeated batch experiments (Fig. 1) a rapid decrease of the enzyme value (12 U/flask) was observed during the first reincubation. After three runs the enzyme yield was 3 U/flask or 12 %. These results are in good agreement with the rapid loss of acid phosphatase activity by free *H. lutea* cells during semicontinuous cultivation (Aleksieva *et al.*, 1998).

*Scanning electron microscopy observations*

The observation of the morphology of the free cells used as a control obtained after 144 h of shake flask fermentation, when the α-galactosidase activity was 24 U/flask or 100 %, was followed. The structure of the free mycelium in this period was typical for filamentous fungi: entangled, intact hyphae were found. In the second cycle of the semicontinuous cultivation (Fig. 1), when the activity decreased 3-fold (8 U/flask), lysis appeared.

Different phases in the development – growth phase (72 h and 144 h), productive phase (second cycle) and phase of deceasing (fifth cycle) – of immobilized *H. lutea* cells were observed by scanning electron microscopy. In the experiments samples with 2.0 g carrier per flask, containing immobilized biomass, were used.

The entrapped spores were precultivated during 144 h under the same conditions as the free cells for the formation of immobilized mycelium (growth phase). The 72 h of precultivation of the sol-gel-entrapped spores marked the beginning of the mycelial growth on the matrix surface: part of the matrix with spreading over the surface cells was clearly seen (Fig. 2A). At the end of the growth

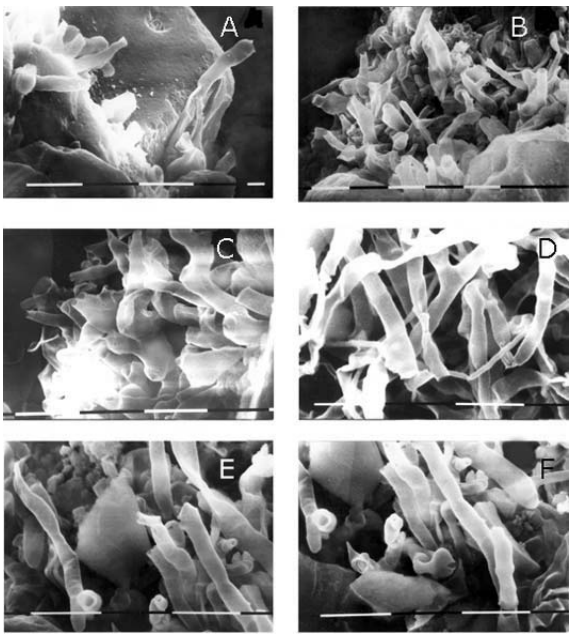


Fig. 2. Scanning electron micrographs of immobilized *H. lutea* cells. Broken vegetative hyphae as a result of vigorous shaking of immobilized mycelium were established. (A, B) Cells in the growth phase (precultivation of the entrapped spores for mycelia formation). (A) Hyphae going out from the matrix grow on its surface (72 h). (B) Intensive growth on the matrix surface (144 h). (C–F) Cell growth in the productive phase (second cycle of semicontinuous cultivation). (C, D) Abundant growth over the whole matrix. (E, F) Mycelia are seen to protrude from the matrix. Bars: 10 μm.

phase (144 h), when the α-galactosidase activity reached 60 % of the control (14.4 U/flask), the mycelium covers almost the whole surface of the hybrid matrix (Fig. 2B). The sol-gel particles containing immobilized biomass were ready for their reuse by means of their transfer into fresh medium at every 144 h. Figs. 2C–F present an intensive fungal growth in the second cycle of the productive phase (Fig. 1) when the α-galactosidase activity sharply increased to reach its maximum (52 U/flask) and exceeded two-fold the activity of free cells.

A dense mycelial network, consisting of vegetative entangled hyphae, was established (Figs. 2C, D). We could scarcely find any zone of the matrix surface that was not covered by the cells (Figs. 2E, F). It was obvious that the immobilized filaments possess good turgur and a smooth surface. This is probably due to the protective role of the carrier that defends the cells from the unfavourable factors of the external environment, as it was previously discussed by Aleksieva *et al.* (2003).

In the following cycles, the enzyme activity started to decrease and in the fifth cycle (phase of deceasing) the α-galactosidase activity was 11 U/flask or 46 % (Fig. 1). The appearance of lysis processes in the culture was in accordance with decreasing of the enzyme activity. Changes in the microbial population appeared. Most of the hyphae were twisted, folded, with torn cell walls and without inner cellular content. On the hybrid matrix surface clusters of cells and hyphae parts existed. In the immobilized hyphae this process was delayed to the fifth cycle where lysed cells were observed. In the free mycelium these processes appeared earlier – in the second cycle.

The presence of massive growth involving intact hyphae, when immobilization in the hybrid sol-gel matrix was carried out, correlates with a 2-fold higher α-galactosidase activity. Such abundant mycelial growth was also observed when *H. lutea* cells were immobilized in a crosslinkable prepolymer for acid proteinase biosynthesis (Aleksieva *et al.*, 1999).

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