Multipurpose application of same immobilized fungous spores

E. Efremenko[1*], O. Spiricheva[1], O. Senko[1], V. Lozinsky[2]
[1] The M. V. Lomonosov Moscow State University, Moscow, Russia
* efremenko@enzyme.chem.msu.ru
[2] The A.N. Nesmeyanov Institute of Organoelement compounds, Russian Academy of science, Moscow, Russia

Introduction

The capability of filamentous fungi for biosynthesis of various products (enzymes, organic acids, etc.) as well as their ability to demonstrate the flexibility in metabolism depending on the content of nutritional medium and conditions of cultivation (temperature, pH, O_2-concentration, etc.) compose a remarkable potential for the development of new polyfunctional biocatalysts of high biotechnological importance. The use of fungus cells in a immobilized form looks very attractive since it allows to shorten the period of accumulation of cell biomass with high metabolic activity and to prolong the period of cell application in the biotechnological processes (Angelova, 2000; Ellaiah, 2004; Nighojkar, 2006; Efremenko, 2006b).
An idea concerning immobilization of filamentous fungus spores with further formation of biocatalyst consisting of immobilized mycelium and polymer carrier seems to be very interesting from biotechnological point of view because this approach enables variations in key properties of developed biocatalytic systems by using same initial materials (cells and carrier) (Fig.1A). The content of nutrition medium used for immobilized mycelium growth can contain various inductors predetermining the development of metabolic processes towards necessary products.

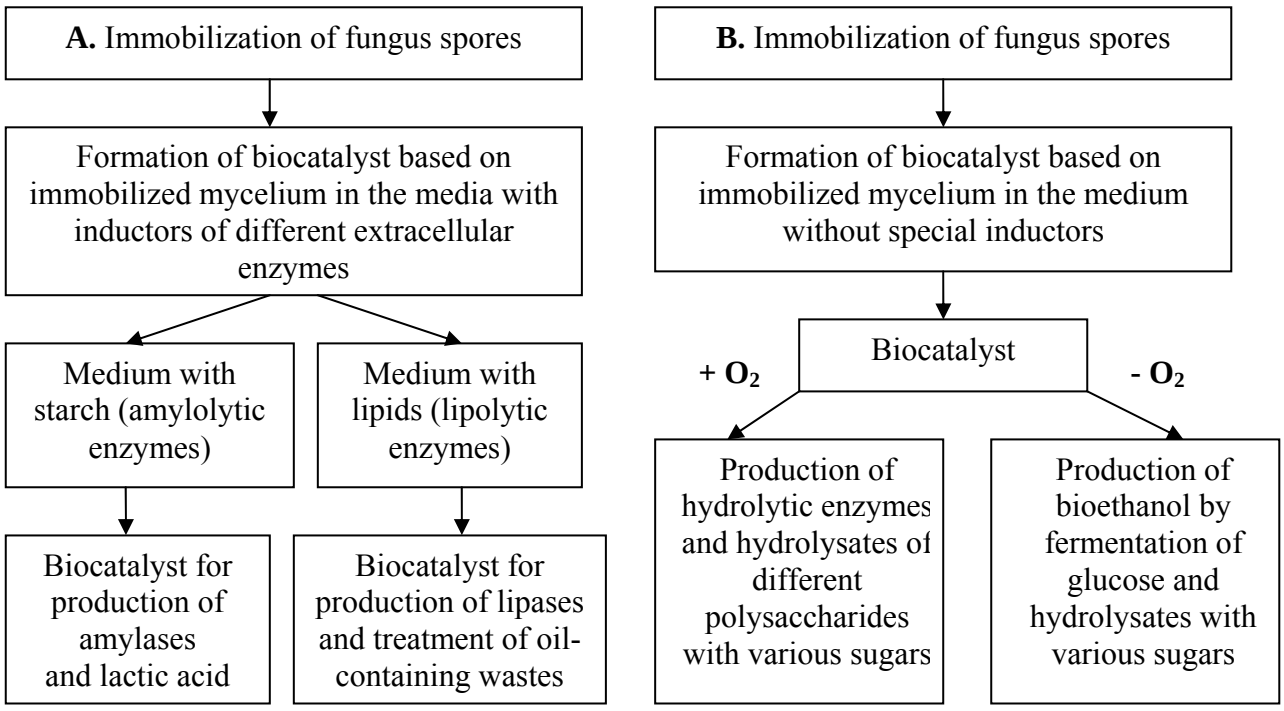

Fig. 1. Examples of schemes illustrating approaches to the obtaining of fungus biocatalysts for the multipurpose application: A) the use of same immobilized spores to form biocatalysts producing different target products and B) the use of formed biocatalyst under dissimilar conditions in the different processes.

Another one attractive approach to the development of multipurpose biocatalysts on the basis of immobilized mycelium might consist in application of same cells entrapped into the carrier in the different processes under various conditions, when the biocatalyst is already formed (Fig.1B). Both approaches were used in the work to investigate the possible multipurpose application of fungus biocatalysts.

Material and methods

The *Rhizopus oryzae*, *Aspergillus terreus* and *Mucor circinelloides* cells were used to prepare the immobilized biocatalysts. All fungus spores were accumulated on potato-dextrose medium (g/L): glucose – 20, $MgSO_4$ – 0.2, $CaCO_3$ – 0.2, potato - 200 g, agar - 20. The cryogel of poly(vinyl alcohol) (PVA) was used as carrier for immobilization of cells.

The biocatalysts with mycelium immobilized into PVA cryogel were obtained using previously patented procedures (Efremenko, 2005; 2006a). In most experiments, the batch application of biocatalysts were carried out in Erlenmeyer flasks with corresponding medium on a shaker Lab-therm (Adolf Kühner, Switzerland) under aerobic conditions with constant agitation (180 rpm) at 28^{o}C. The media were refreshed after each working cycle.

The media with glucose, acid hydrolysate of starch and gelatinized starch for the LA fermentation were prepared as published previously (Efremenko, 2006b). Starch concentration was analyzed by iodine colorimetric method. Concentration of L(+)-lactic acid (LA) and glucose were assayed by the enzymatic methods using L(+)-lactate oxidase-peroxidase kit (Sentinel, Italy) and glucose oxidase-peroxidase kit (Impact, Russia), respectively.

The investigation of biosynthesis of secreted lipases by immobilized fungus mycelium was carried out in the following medium (g/L): corn steep liquor - 40; peptone – 10, KH_2PO_4 – 14, K_2HPO_4 – 2.4, $MgSO_4$ – 0.4. The corn oil or margarine (each per 1% w/w) was introduced to the cultural medium as main sources of lipids. The amount of enzyme catalyzing the hydrolysis of 1 μmole substrate (tributyrin, Vekton, Russia) for 1 min at 40°C and pH 6.5 was accepted as a unit of lipolytic activity. The specific activity was related to 1 L of cultural medium.

In the experiments aimed at the fermentation of various sugars by immobilized fungus cells, the sugar concentrations were determined using a Shimadzu LC-9A HPLC system (Japan). The following medium was used for the investigation of fermentation under anaerobic conditions (g/L): sugar - 50; yeast extract - 2.0; NaCl - 1.0; $(NH_4)_2SO_4$- 2.0; $MgSO_4x7H_2O$ - 1.0; KH_2PO_4- 13.5 (pH 6.8). Ethanol concentration was analyzed using gas liquid chromatography (Hewlett-Packard 5880, USA). The theoretical yield of ethanol from hexoses, pentoses, di- and trisaccarides was calculated as described by Christakopoulos (1989).

Results and Discussion

The use of same spores of *R. oryzae* cells entrapped into PVA cryogel for the formation of active biocatalysts in the different media enabled obtaining of immobilized mycelium with absolutely different features (Fig.2, Tab.1). It was shown that cultivation of immobilized spores in the medium with various lipid sources allowed to obtain biocatalyst with stable synthesis of extracellular lypases (Fig.2). According to obtained results, margarine, containing more residues of saturated fatty acids in the lipid content compared to corn oil, induced appearance of slightly higher lipolytic activity in 1 L of medium with immobilized mycelium.

The application of media with glucose added as pure compound or as part of acidic starch hydrolysates enabled the obtaining of biocatalyst with high LA-productivity and improved resistance to the high concentrations of last product accumulated in the cultivation medium (Table 1). The possibility of long-term and effective use of biocatalyst based on the immobilized fungus cells secreted amylolytic enzymes in the direct production of LA from starch was demonstrated.

It was established that both biocatalysts obtained using same immobilized spores of *R. oryzae* retained their metabolic activities in the corresponding processes for a long time (at least 25 days).

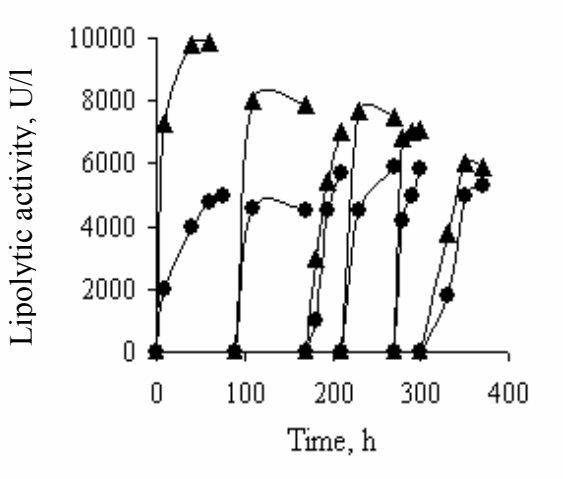

Fig. 2. Lypolytic activity determined in the cultural medium during the long-term batch process of lipase production by immobilized *R. oryzae* cells. The cultivation of immobilized cells was realized with corn oil (●) and margarine (▲) as main sources of lipids in the cultural medium.

It is known that hexoses can easily be fermented by yeast cells but the fermentation of pentoses is more difficult process. Several economic evaluations showed that efficient fermentation of pentoses is important for the overall economy of ethanol production from lignocellulosic materials (Nguyen & Saddler, 1991). The cellulolytic enzymes of filamentous fungi are widely used to obtain the hydrolysates of lignocellulosic materials. It appeared that some filamentous fungi can not only hydrolyze lignocellulosic materials providing glucose, xylose and cellobiose under aerobic conditions but also fermented various sugars to ethanol under anaerobic conditions (Christakopoulos, 1989; Pushalkar, 1998).

In this work, two biocatalysts were prepared on the basis of fungus strains *A. terreus* and *M. circinelloides* producing cellulolytic enzymes by immobilization of spores into PVA cryogel with further formation of immobilized mycelium (Fig.1B). It was established that both biocatalysts possessed high cellulolytic activity under aerobic conditions (data not shown) and could ferment a wide spectrum of various sugars with high enough yield of ethanol under anaerobic conditions (Fig.3).

Initial substrate concentration, g/L	Maximal concentration of LA, g/L	Yield of LA, %
Glucose		
120 [a]	113 ± 4	94 ± 4
120 [b](297*)	184 ± 6	62 ± 2
Acidic hydrolysates of starch		
110 [a]	57 ± 5	52 ± 4
110 [b] (244*)	139 ± 3	57 ± 1
Gelatinized starch		
50	13 ± 2	26 ± 4
70	15 ± 2	21 ± 3

Table 1. Characteristics of process of LA-production with immobilized *R. oryzae* cells: batch process (a) and semi-batch process with substrate additions (b). Symbol * marks the total amount of introduced glucose (g/L).

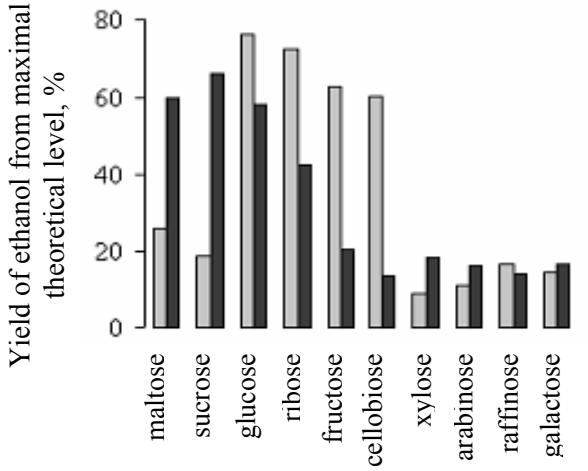

Fig. 3. The yield of ethanol from maximal theoretical level in the fermentation of various substrates by filamentous fungus cells *M. circinelloides* (grey columns) and *A. terreus* (black columns) immobilized into PVA cryogel.

Conclusions

The use of same spores of filamentous fungous cells immobilized in PVA cryogel for the obtaining of biocatalysis producing various target products was demonstrated. The type of substrate played the main role in the formation of biocatalysts with certain metabolic activity. The use of the same fungus biocatalyst in the various processes (enzyme biosynthesis and fermentation) was established.

Acknowledgement

The work was financially supported by Russian Foundation of Basic Research (Project ofi_a 05-04-08018) and federal Agency of Science and Innovations of Russian Federation (State contract No. 02.434.11.3005).

References

Angelova M.B. et al. (2000) Comparison of antioxidant enzyme biosynthesis by free and immobilized *Aspergillus niger* cells. *Enz. Microb. Technol*., V.26, p.544-549.

Christakopoulos P. et al. (1989) Direct fermentation of cellulose to ethanol by *Fusarium oxysporum*. *Enz. Microb. Technol.*, V.11, p. 236-239.

Efremenko E.N., et al. (2005) Immobilized biocatalyst, method of its preparation and method of lactic acid production with the use of this biocatalyst. *Patent RU No. 2253677*.

Efremenko E.N., et al. (2006a) Immobilized biocatalyst for biological purification of fat-containing wastewaters and the method of biocatalyst production. *Patent RU Proposal No. 2006107637.*

Efremenko E.N., et al. (2006b) L(+)-lactic acid production using PVA-cryogel entrapped *Rhizopus oryzae* fungus cells. *J. Chem. Technol. Biotech.*, V.81, p. 519-522.

Ellaiah P. et al. (2004) Production of lipase by immobilized cells of *Aspergillus niger*. *Process Biochem*., V.39, p.525-528.

Nguyen Q.A., Saddler J.N. (1991) An integrated model for the technical and economic evaluation of an enzymatic biomass conversion process. *Bioresour. Technol.*, V.35, p.275-282.

Nighojkar S. et al. (2006) Production of polygalacturonase by immobilized cells of *Aspergillus niger* using orange peel as inducer. *Process Biochem*., V.41, p.1136-1140.

Pushalkar S. and Rao K.K. (1998) Short communication: ethanol fermentation by a cellulolytic fungus *Aspergillus terreus*. *World J. Microb. Biot*., V.14, p.289-291.