Effect of temperature on enzyme activity of immobilized microbial cells

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Introduction

Immobilized biocatalysts are more stable ones and can be applied in synthetic or degradation processes, including remediation of contaminated sites (Dias et al. 2000; Soares et al. 2006). Different agents like temperature and toxic chemicals promote biocatalyst inactivation. Being in an immobilized state the living cells, producers of thermostable enzymes are in a way covered by the microenvironment of the carrier used that defends them from such unfavorable effects. Nitriles are highly toxic substrates that are found as dangerous pollutants of the environment as a result of different industrial processes. There exists an urgent need for developing of procedures that can reduce the nitrile concentrations throughout the environment because their effect is harmful and disturbing. Employing immobilized microorganisms producing nitrile-metabolizing enzymes could be an effective way for detoxification of contaminated waters and soils.
The sol-gel technique allows the utilization under mild biocompatible conditions of porous matrices that present several advantages over the polymeric matrices used for biomolecule immobilization (Alvares et al. 2006 Desimone et al. 2005). They are chemically inert and have great mechanical and thermal resistance. They are not affected by contact with organic solvents or microbial attack as well as could present optical transparency (Brinker et al. 1990).
Soybean meal (SBM) is the protein supplement most frequently used in animal feed worldwide. However, SBM is not without limitations because of oligosaccharides such as raffinose and stachyose that are not digested in the intestines of monogastric animals (Gitzelmann et al., 1965). They cannot synthesize sufficient α-galactosidase (EC 3.2.1.22) in their intestinal systems to hydrolyze the α-galactosides present in soybeans and other legumes. These disturbances reduce feed efficiency in monogastric animals (LeBlanc et al., 2005). To improve the nutritional quality of SBM for monogastric animals, α-galactosidase is generally used to reduce the level of raffinose series oligosaccharides. α-Galactosidase is known to occur widely in microorganisms, plants (Kang et al. 2001) and humans (Gitzelmann et al, 1965). Various microorganisms produced α-galactosidase, such as fungi (Wang et al., 2004), yeasts (Oda et al., 1996) and bacteria (Jin et al., 2001).
Immobilization of the fungal producer of α-galactoside promotes its secretion and increases the enzyme level for a long period.
The aim of the present study was to investigate the effect of temperature on the enzyme activity of the cells of both strains, immobilized in sol-gel hybrid matrices on the basis of TEOS with the addition of PEG and PVA.

Material and methods

The following chemicals have been used: TEOS (Merck), PEG (MW 400), PVA (MW 82000), 0.1 N HCl, phosphate buffer with pH=7.2 at 20◦C.

The chemical conditions of the sol-gel method were slightly modified in order to fit the requirements of the biochemistry. No alcohol was added as a co-solvent to prevent protein denaturation. The precursor solution was vigorously mixed until the initially turbid mixture became a clear solution. Phosphate buffer (0.06 M) with a pH 7.2 was added to the hydrolyzed precursor solution and pH was raised up to neutral. The hybrid inorganic-organic gels were prepared by replacing 20% of TEOS with PEG and PVA in the acidic precursor solution. The gelation time was less than a minute after the addition of cell suspension. Thin, transparent hybrid flakes were obtained. This is followed by an overnight drying.

The bacterial strain *Bacillus sp*. UG-5B (№8021) was deposited in the National Bank for Industrial Microorganisms and Cell Cultures, Bulgaria.

The nitrilase activity assay was realized by measuring the ammonia released, following the phenol-hypochloride method of Fawcett and Scott (1960). One enzyme unit is defined as the amount of enzyme producing 1 μmol ammonia min^{-1} at pH 7.2, 45◦C and 20mM benzonitrile as a substrate.

The concentration of microbial cells is determined by the optical density at 660 nm and calculated as mg biomass ml^{-1} according to a standard curve in relation to dry weight.

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

α-Galactosidase activity was assayed (Dey et al., 1993). The amount of the released p-nitrophenol was measured from 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 minute under the described conditions.

Six milliliters of the spore suspension (10^{10} spores/ml) were entrapped in the matrix. The washed and dried sol-gel pieces with the entrapped spores were precultivated for 144h in 500 ml Erlenmeyer flasks with 50 ml medium in a rotary shaker (220 rpm) at 30°C and 35°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.

Results and Discussion

The sol-gel derived hybrid silica nanocomposites were employed as immobilization supports to produced active biocatalysts and use them in batch processes at 30°C, 35°C 50°C, 55°C, 60°C, 65°C. The nitrilase activity of immobilized bacterial cells of *Bacillus sp*. UG-5B was followed to establish that the increase in temperature led to increased activity and the maximum appeared to be at 60°C (2.25 U) (Fig. 1).

It is known that *Humicola lutea* is a mesophilic fungus (Bertoldi et al., 1976), we carried out the cultivation at 35°C. The sharp increase in the α-galactosidase yield up to 1.9 $U.ml^{-1}$ after cultivation at 35°C demonstrated the temperature effect and the importance of all fermentation factors for industrial development of enzyme production under low cost using soy meal extract as a nutrient medium. For this reason the comparative experiments for cultivation of the immobilized mycelium at 30°C and 35°C was performed. Fig.2 presents the α-galactosidase activity by encapsulated in a hybrid sol-gel matrix *H. lutea* cells during repeated batch shake flask cultivation at these temperatures. It is clearly seen that in the first and second cycles the enzyme yield at 35°C is about 50% higher in comparison to the one at 30°C or 2.6 fold higher as compared to the free cell fermentation. Half-life time of α-galactosidase production was 4 cycles or 24 days for both cultivation temperatures.

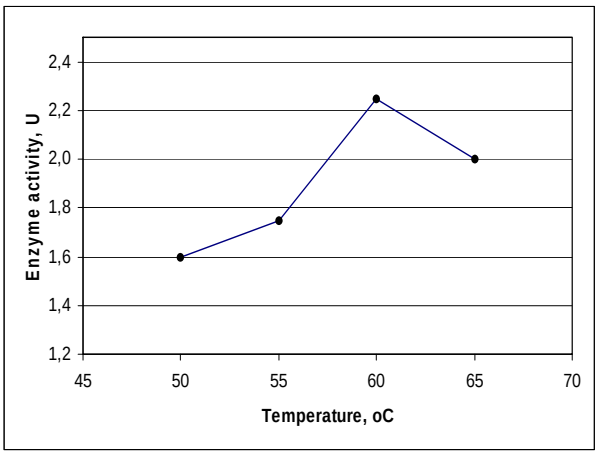

Fig. 1 Nitrilase activity at different temperatures.

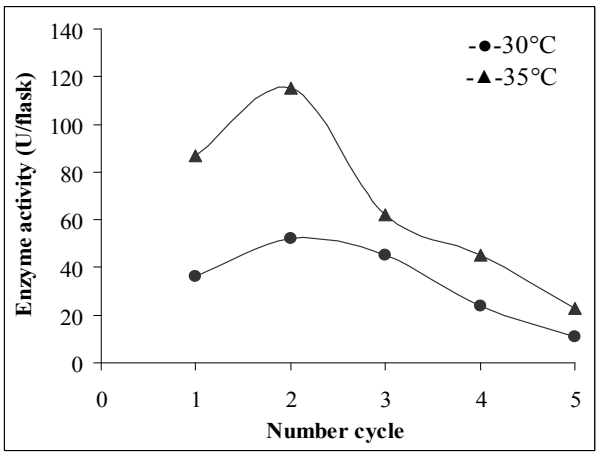

Fig. 2 α-Galactosidase activity in relation to temperature for 5 batch cycles.

Scanning electron microscopy examination of the *H. lutea* immobilized mycelium used for 12 days (two runs) was presented in Fig. 3. The observations confirmed that the dense mycelial network on the matrix surface was composed of thick and viable hyphae when the maximal enzyme yield (115 U per flask or 260%) was reached (Fig. 2). After five successive batches (30 days) - Fig. 4 in the semicontinuous cultivation when the enzyme activity decreased to 23 U per flask or 52 % as compared to free cells.

Fig.3 SEM of vegetative *H. lutea* cells covering the sol-gel matrix in the second batch at 35°C. Bar= 10µm.

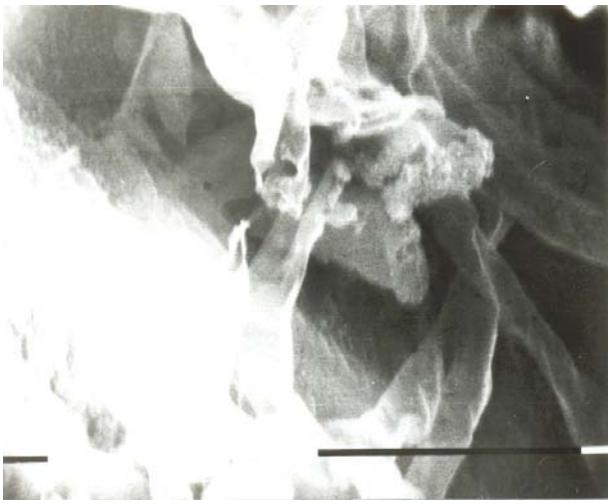

Fig.4 SEM of the beginning of lysis and presence of hollow cells in the deceasing face. Bar=10µm.

Electron transparent and lysed hyphae were found. Similar growth behavior of in the productive phase as well as in the deceasing phase was observed using *H. lutea* mycelium immobilized in polyhydroxyethylmetacrylate gel (Aleksieva et al., 2007) for acid phosphatase production or in crosslinkable prepolymer for acid proteinase biosynthesis (Aleksieva et al., 1999).

Conclusions

At successive batch cycles and increasing the temperature to follow the α-galactosidase production by immobilized mesophilic fungus *Humicola lutea,* a higher enzyme level was obtained (102%-260%) compared to free cells. The same matrices which appeared to be inert and stable and suitable to promote a high nitrilase activity at entrapment of thermophilic *Bacillus sp.* cells at 50°C, 55°C, 60°C, 65°C reached a maximum at 60°C. The biocompatibility and the high surface area, make the sol-gel hybrid material, synthesized on the basis of TEOS and PEG+PVA promising for encapsulation of cells. Electron microscopy observations on the fungal growth and enzyme activity measurements for both investigated enzymes showed that these matrices provide a suitable platform for immobilization of fungal and bacterial cells. They can be used and reused without disruption.

Acknowledgements

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References

1. A. Soares et al., (2006) *Biodegradation of nonyphenol in a continuous bioreactor at low temperatures and effects on the microbial population.* Appl Microbiol Biotechnol 69 597-606
2. C. Brinker et al., (1990) *The physics and chemistry of sol-gel processing*. Sol-gel Science, Academic Press, San Diego, CA
3. C. Wang et al., (2004) *Influence of cultivating conditions on the α- galactosidase biosynthesis from a novel strain of Penicillium sp. in solid-state fermentation.* Lett Appl Microbiol 39 369-375
4. F. Jin et al. (2001) *Thermostable α-amylase and α-galactosidase production from the thermophilic and aerobic Bacillus sp. JF strain.* Process Biochem 36 559-564
5. G. Alvarez et al., (2006) *Immobilization of bacteria in silica matrices using citric acid in the sol-gel process.* Applied Microbiology and Biotechnology 18 1-9
6. H. Kang et al. (2001) *Characteristics of an α-galactosidase associated with grape flesh.* Phytochemistry 58 213-219
7. J. Dias et al., (2000*) Biodegradation of acetonitrile by cells of Candida guilliermondii UFMG-Y65 immobilized in alginate, k-carrageenan and citric pectin.* Brazilian J of Microbiol 31 61-66
8. J. Fawcett et al. (1960) *A rapid and precise method for determination of urea.* J Clin Path 13 156-159
9. J. LeBlanc et al. (2005) *Lactobacillus fermentum CRL 722 is able to deliver active α-galactosidase activity in the small intestine of rats.* FEMS Microbiol. Lett. 248 177-182
10. M. Bertoldi (1976), *New species of Humicola: an approach to genetic and biochemical classification.* Can J Botany 54 2755-2768
11. M. Desimone et al., (2005) *Efficient preservation in silicon oxide matrix of Escherichia coli, producer of recombinant proteins.* Appl Microbiol Biotechnol 68 747-752
12. P. Aleksieva et al. (1999) *Crosslinkable prepolymer-immobilized Humicola lutea for acid proteinase biosynthesis*. Microbiol Res 154 205-211
13. P. Aleksieva et al. (2007) *Production of extracellular α-galactosidase by mesophilic filamentous fungi during submerged cultivation.* Compt Rend Acad Bul Sci 60 691-696.
14. P. Dey et al. (1993) *Induction of alpha-galactosidase in Penicillium ochrochloron by guar (Cyamopsis tetragonobola) gum.* Biotechnol Appl Biochem 17 361-371
15. R. Gitzelmann et al. (1965) *The handling of soy alpha-galactosidase by a normal and galactosemic child.* Pediatrics 36 231-235
16. Y. Oda et al. (1996) *α-Galactosidase from the yeast Torulaspora delbrueckii IFO 1255*. J Appl Bacteriol 80 203-208