Phenol Biodegradation by Fungal Cells Immobilized in Sol-Gel Hybrids

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The capability of cells of the fungus *Aspergillus awamori*, either free or immobilized in hybrid sol-gel material cells, for phenol biodegradation was demonstrated. Phenol was present in the reaction mixture as the sole carbon and energy source, and its decomposition was followed in repeated batch degradation experiments. Atomic force microscopy provided information on the development of self-organizing structures in the materials synthesized by the sol-gel method. Phenol biodegradation was mediated only by the fungal cells, and no absorption by the hybrid matrix was observed. Ten cycles of phenol biodegradation using the immobilized cells system were conducted during which up to 2000 mg l^{-1} phenol was completely decomposed. Immobilized cells degraded phenol at 8.33 mg h^{-1}, twice as fast as free cells. The good performance of the immobilized fungal cell system is promising for the development of an efficient technology for treating phenol-containing waste waters.

Key words: Aspergillus awamori, Phenol Biodegradation, Sol-Gel Hybrid

Introduction

Aromatic compounds are widely used in industrial and agricultural activities and are often discharged into the environment during their use. Phenol and its derivatives are typical aromatic compounds that occur widely in wastes released into rivers and soils and even into the atmosphere. Taking in mind the wide spectrum of pollutants and their increase in the environment, the potential of some microorganisms to degrade phenolic compounds is of considerable interest and importance with impact to nature protection (Diaz, 2004; Quintelas *et al*., 2006; Alexieva *et al*., 2008; Hristov *et al*., 2010).

Many treatment techniques have been employed in the past few years to reduce the concentration of phenol in the environment, including biodegradation, adsorption, ion exchange, and the use of bioactive activated carbon (Pathade *et al*., 2001). Biological treatment has proven to be the most promising and effective method for the removal of phenol from waste water, leading to complete mineralization of phenol within a wide range of concentrations (Li *et al*., 2006; Tsekova *et al*., 2011).

Filamentous fungi belonging to the genera *Penicillium*, *Aspergillus*, *Fusarium*, and *Graphium* have been cited for their potential to degrade phenol (Santos and Linardi, 2004; Stoilova *et al*., 2007; Yemendzhiev *et al*., 2009). There are only few reports on phenol degradation by immobilized microbial cells in different materials (El-Naas *et al*., 2009; Jordanova *et al*., 2009; Branyik *et al.*, 2000; Branyik and Kuncova, 1998), but there are no data available on the entrapment of cells of filamentous fungi in hybrid sol-gel materials and their application to waste water treatment and phenol removal. Immobilized cells have some advantages over free biomass due to the possibility of their multiple use and application for an extended period of time, being protected from the high phenol concentrations, as well as the ease of separation and reutilization of the immobilized biomass. From this point of view, immobilization of fungal cells in hybrid matrices synthesized by the sol-gel method is very promising for practice.

Composites including organic and inorganic components are of special interest as they have characteristics in between the two original phases or even attain new features. Organic-inorganic

hybrids can be formed in various combinations of metal alkoxides and polymers to create a nanoscale mixture of inorganic oxides and organic polymers by the sol-gel method (Niepceron *et al*., 2009; Shchipunov *et al*., 2004). Hybrids, composed of inorganic oxides covalently bound to organic polymers, are of special interest, due to the lack of interface imperfections. The porous structure of the hybrids is a major parameter for the efficiency of the immobilization process. The present study investigates the immobilization of *Aspergillus awamori* spores in hybrid matrices with chitosan and explores their ability to biodegrade phenol during a repeated batch process.

Material and Methods

Microorganism and medium

The strain of the fungus *Aspergillus awamori* from the Collection of the Institute of Microbiology of the Bulgarian Academy of Sciences, Sofia, Bulgaria, was used in the present study. It was isolated from soil samples taken near an industrial plant for glucose and starch production. The strain was adapted to phenol by gradually raising the concentration of the xenobiotic from 50 to 200 mg l^{-1} in 2% (w/v) malt agar. The spores obtained during cultivation on the highest phenol concentration were maintained on the same medium at 28 °C for 7 d to obtain dense sporulation.

Modified Chapek-Dox liquid medium with 20 g l^{-1} and 200 g l^{-1} phenol, respectively (pH 6.0), was used as medium for spore germination and mycelium formation. The same medium without carbohydrate but rather phenol as the sole carbon and energy source was used for the phenol biodegradation process by both free and immobilized cells.

Sol-gel synthesis

The silica-chitosan hybrids were prepared through the sol-gel method. Tetraethylorthosilicate (TEOS) was prehydrolyzed with water and ethanol, and hydrochloric acid was used as catalyst (1 ml, 0.1 M). Chitosan solutions were prepared by step-wise addition of the sample to acetic acid and homogenization on a magnetic stirrer. After dissolution, the two previous solutions were mixed with the prehydrolyzed TEOS. The molar ratio TEOS/H_2O/C_2H_5OH/HCl was $1:2:8:1 \cdot 10^{-3}$. The content of silica was kept constant, and hybrids containing 5, 10, 20, and 40 wt.-% of the organic material with respect to SiO_2 were prepared.

Immobilization procedure and culture conditions

Ten ml of spore suspension (10^6 spores ml^{-1}) were entrapped in the sol solution. The dried pieces with the entrapped spores were pre-cultivated in 500-ml Erlenmeyer flasks with 100 ml growth medium in a rotary shaker (150 rpm) at 28 °C until depletion of both carbon sources. The same operation was carried out with the free spores as a control. Then the washed particles containing the immobilized biomass as well as the free mycelium were ready for use as an inoculum in the repeated batch cycles for phenol biodegradation.

Repetition of the batch culture

The phenol biodegradation process during repeated use of the immobilized biomass in sequential batch cultures was investigated using samples of the immobilized mycelium prepared from 2 g hybrid matrix per flask. Repeated batch experiments were carried out in duplicate under the conditions described above. At the end of each batch, both immobilized mycelium and free biomass (as a control) were washed and transferred into fresh medium to start a new run. The culture filtrates were assayed for residual phenol concentration. During the experiments with immobilized cells, the deviation between replicates was less than 2%, whereas in the case of free cells, a deviation of 4% was observed.

Analytical methods

Phenol concentrations (in mg l^{-1}) were determined colorimetrically with 4-aminoantipyrine according to Greenberg *et al*. (1992).

Methods for investigation of the hybrid material

Atomic force microscopy (AFM) images were created using a Digital Instruments multimode atomic force microscope equipped with a nanoscope IIIa controller (Digital Instruments, Santa Barbara, CA, USA). The results were obtained in the tapping mode AFM. A vertical engage 4842 JV scanner (Digital Instruments) and Si probes were applied in all experiments. The driving frequency in the tapping mode was chosen at the resonant frequency of the free-oscillating cantilever in the immediate vicinity of the sample

surface. Height and phase images were recorded simultaneously.

The average roughness (*Ra*) of the hybrid surface was calculated directly from the AFM image.

BET (Brunauer, Emmett, Teller) N_2 adsorption at 77 K was utilized to determine the specific surface areas and porosities of the prepared hybrids. A gas adsorption manometric apparatus (ASAP-2020 analyzer; Micromeritics Instrument Corporation, Norcross, GA, USA) was used for the N_2 adsorption experiments. The BET equation (Brunauer *et al.*, 1938) was used for the calculation of the specific surface area.

Results and Discussion

Matrix investigations

Adsorption/desorption isotherms of the silica-chitosan hybrids are presented in Fig. 1. The isotherms can be classified as a type IV isotherm with a H_2 hysteresis, which illustrates the mesoporosity of this material. Indeed, the mechanism of the formation of this mesoporous material is dictated by two features. The first is the dynamics of the surfactant molecules to form molecular assemblies which lead to micelles, and the second is the ability of the inorganic oxide to undergo condensation reactions to form thermally stable structures.

More detailed information on the nanostructure of the matrices is obtained from the AFM studies. They reveal the evolution of the self-organizing structures in the synthesized materials. Good coincidence was found between the sizes of nanoparticles (from 7 to 12 nm) and nanoaggregates (about 40–50 nm) observed by AFM. It was established that all samples have surfaces with irregularities of quite small height (Table I). For the hybrids containing TEOS and 5% chitosan, the largest observed heights were 1.9 nm.

Phenol degradation by free and immobilized cells

Based on the results obtained by BET and AFM roughness analyses, we chose a hybrid matrix containing 40% chitosan because for better growth and development of the fungal strain a larger surface area and higher average pore size are appropriate (Spasova *et al.*, 2008). Chitosan has previously been found a biocompatible and favourable additive in the matrix (Singh *et al.*, 2010).

Degradation of phenol by free and immobilized *Aspergillus awamori* cells was investigated under conditions of repeated batch cultivation. The initial concentration of the xenobiotic was 200 mg l^{-1} at the beginning of each cycle. The results are presented in Figs. 2a and b.

In the case of free cells, degradation of 600 mg l^{-1} phenol was achieved within a period of 13 days, while immobilized cells degraded 2000 mg l^{-1} phenol in 10 successive cycles within 10 days at a maximal rate of 8.3 mg h^{-1}, which was more than twice that observed for the free mycelium (Fig. 3). A similar behaviour was observed by Passos *et al.* (2010) who compared phenol degradation by free and encapsulated cells of a newly isolated *Aspergillus* sp. strain. This indicates the presence of a favourable protective microenvironment inside the sol-gel matrix which reduces the abiotic stress of the entrapped cells. On the other hand, Santos *et al.* (2003) reported higher rates of degradation of up to 12 mM phenol by free cells of the strain *Graphium* sp. FIBY, due to limitations to substrate diffusion in the alginate matrix. However, in our hands, immobilization of *Aspergillus awamori* cells in a hybrid sol-gel matrix with chitosan resulted in a performance better than that of free cells in the repeated batch process by reducing the time for complete phenol degradation (Figs. 1a and b). Entrapped cells rapidly degraded the phenol for 24 h, while for the free cells an adaptation period of 6 days was observed during the third cycle which was due to the strong inhibitory effect of phenol. After the adaptation time the phenol concentration decreased slowly for the next 48 h. No differences were observed between the first two cycles of phenol biodegradation. For the immobilized culture, the carrier material acts as a protective cover against the toxicity of phenol, and the gel network serves as a diffusion barrier for phenol that is lacking in the free cell culture (Chen *et al.*, 2002).

In order to distinguish between phenol absorption by the sol-gel matrix and phenol biodegradation by the fungal cells, the pure carrier material was tested for its adsorption of phenol. For this purpose, 2 g of sol-gel matrix without entrapped cells were immersed in 200 mg l^{-1} phenol solution for 24 h, during which the phenol concentration was not found to significantly change (data not shown). Thus the reason for the decrease in the phenol concentration during cultivation was its biodegradation by the immobilized cells.

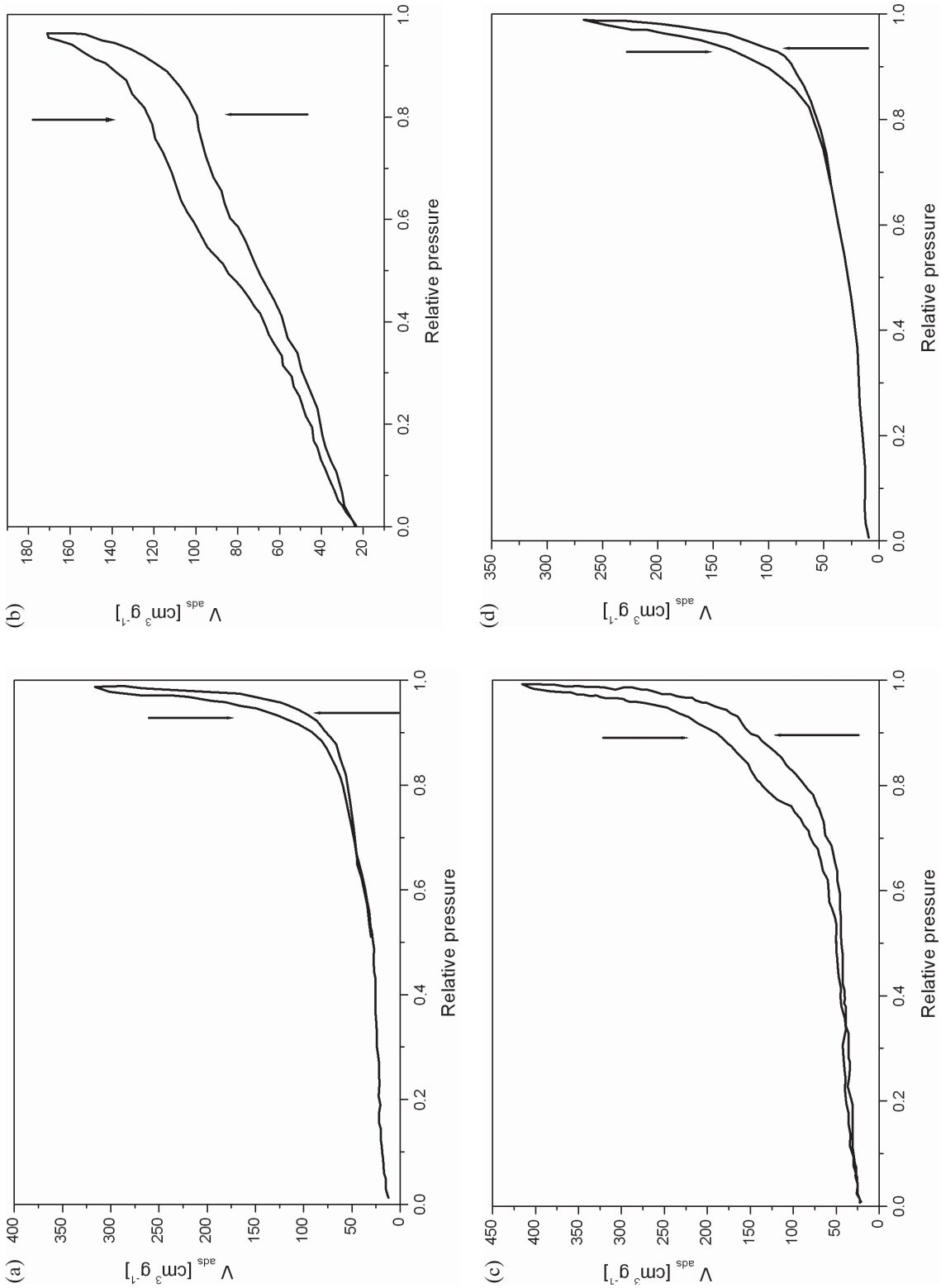

Fig. 1. Typical adsorption (↑)/desorption (↓) isotherms of nitrogen physisorption on silica-chitosan hybrids: (a) 5% organic part; (b) 10% organic part; (c) 20% organic part; (d) 40% organic part. Results represent average values from triplicate measurements.

Table I. Roughness parameters.

Sample	RMS roughness [nm]	Average height [nm]	Maximal height [nm]
TEOS + 5% chitosan	0.2293	0.4267	2.1371
TEOS + 10% chitosan	0.4427	0.9147	2.5620
TEOS + 20% chitosan	0.4769	1.2824	3.5639
TEOS + 40% chitosan	0.5549	1.7232	4.2659

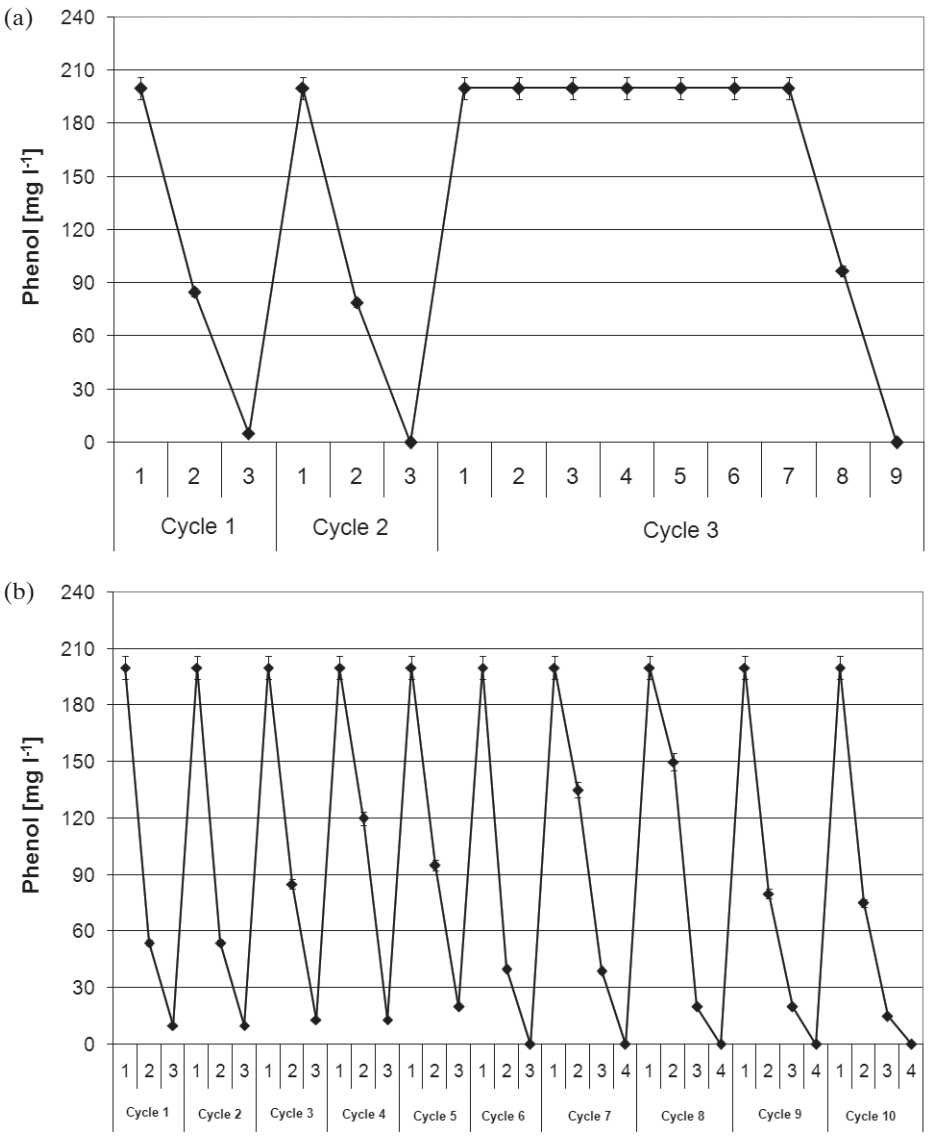

Fig. 2. Phenol concentrations in repeated batch degradation experiments employing (a) free and (b) immobilized cells of *Aspergillus awamori*. Results represent average values from triplicate measurements. Each sample is given by a number.

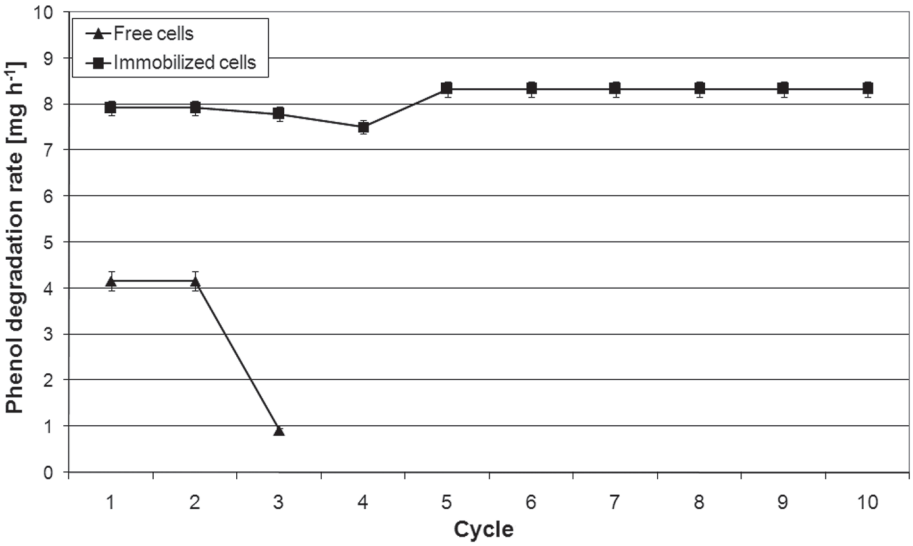

Fig. 3. Rates of phenol biodegradation by free and immobilized *Aspergillus awamori* cells during repeated batch cultivation.

The use of biological systems for bioremediation is more cost-effective than the traditional cleaning techniques. Most of the studies carried out so far have depended on the use of free cell systems, but procedures involving cell immobilization can be a better alternative. There is a great potential of immobilized cells in industrial and biotreatment applications (Cheetham, 1980). The cells entrapped in a suitable and biocompatible matrix under optimal conditions remain viable for a long time and thus are a better choice than free cell systems. *Aspergillus awamori* entrapped in a hybrid sol-gel matrix has proven useful for its biotechnological application in the treatment of phenol-containing effluents. The obtained data (Fig. 1) indicate that the rate of degradation of phenol was enhanced in the immobilized system that can be continuously used for periods of more than 10 days without any loss of its biodegradative capacity.

Conclusion

A novel immobilized system on the basis of hybrid sol-gel matrices with chitosan and *Aspergillus awamori* cells was established for phenol biodegradation and found to be more efficient and stable than free cells. Thus the microbial immobilization technology becomes an extremely versatile approach in the detoxification of industrial effluents containing toxic organic pollutants.

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