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ORIGINAL PAPER

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Immobilization of cells with nitrilase activity from a thermophilic bacterial strain

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Abstract Cells of the moderately thermophilic *Bacillus* sp. UG-5B strain, producing nitrilase (EC3.5.5.1), which converts nitriles directly to the corresponding acid and ammonia, were immobilized using different types of matrices and techniques. A variety of sol-gel silica hybrids were tested for entrapment and adsorption of bacterial cells as well as chemical binding on polysulphone membranes. Activation of the matrix surface with formaldehyde led to an increase in immobilization efficiency and operational stability of the biocatalysts. Among the supports screened, membranes gave the best results for enzyme activity and especially operational stability, with retention of 100% activity after eight reaction cycles.

Keywords Immobilization · Thermophilic bacterium · Nitrilase production

Introduction

The use of microorganisms to degrade environmental pollutants is emerging as an alternative technology for restoring contaminated sites. Nitriles are pollutants of soils and industrial waste waters with a wide distribution in the environment [15]. They occur naturally in plants and as intermediates in microbial metabolism [5, 13, 14], and also as products of the petrochemical industry and waste treatment, and are widely used as chemical solvents, pesticides, recrystallizing agents and chiral synthons. Nitriles are toxic due to their cyano group (R-CN). This is the reason for the considerable commercial interest in nitrile-degrading enzymes [3, 12]. These versatile biocatalysts have potential applications in different fields including bioremediation of soils and waters [1]. Mesophilic bacteria are the main producers of these enzymes. To employ more stable enzymes at higher temperatures is very important. There are few reports on thermostable bacterial nitrilases [6]. A variety of methods for the immobilization of microbial cells are known, which simplify catalyst recovery and reuse, improve the resistance of cells to lysis, and increase the stability of the enzyme activity of the immobilized cells and effectiveness of the degradation process. Nitrilase-producing bacteria have already been immobilized on polyvinyl alcohol (PVA) [2], DEAE cellulose [10], alginate [11], and in polyacrylamide and carrageenan gels [4]. However, there have not been many studies on immobilization of nitrilase-producing thermophilic bacteria.

This work focused on screening of different carriers and methods for immobilization of *Bacillus* sp. UG-5B cells to obtain a stable biocatalyst, suitable for application over a wide temperature range.

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Materials and methods

Microorganism and medium

The Gram-positive, sporulating, moderately thermophilic strain *Bacillus* sp. UG-5B [8] was isolated from polluted industrial waters by our group after screening procedures. This strain was deposited in the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC—Bulgaria) no. 8021/2001. Cells of this strain were used in the immobilization procedures.

A stirred fermentor Bioflo (New Brunswick Scientific, Edison, N.J.) with a working volume of 350 ml was used. Cultivation was carried out at 50°C with an air flow rate of 1.0 vvm. Fermentation medium contained

(g/l): peptone 5.00 (Oxoid); yeast extract 5.00 (Oxoid); K_2HPO_4 5.00; $MgSO_4$ 0.10; NaCl 1.00; $FeSO_4 \cdot 7H_2O$ 0.03, and 20 mM benzonitrile, pH 7.5. Cells were separated from the culture medium in their stationary state by centrifugation and resuspended in a phosphate buffer solution (0.06 M, pH 7.2). Cell suspension with a concentration of 15 mg/ml and enzyme activity of 2.25 U/ml was used for immobilization.

Immobilization procedures

Matrices

Polysulphone membranes (Membrane Technologies, Plovdiv, Bulgaria) with pore size 0.45-μm were used as carriers. Different hybrids of sol-gel matrices were synthesized and supplied by the group of B. Samuneva [16]. The SiO_2 precursor in the matrices was substituted with 5–20% of organic components such as polyethylene glycol (PEG), PVA or a combination of the two.

Methods

Two types of immobilization for the organically modified silica matrices were applied: entrapment and adsorption. When entrapment was performed, 5 ml cell suspension was added to the sol-gel volume (40 ml) before gelation. Gelation takes place within less than 1 min. Dry biocatalysts (4 g) were obtained after 24 h drying on Petri dishes at room temperature. Adsorption on the prepared hybrid silica carriers was carried out. Cell suspension (5 ml) was used for immobilization on 2 g carrier. Immobilization took place overnight on a rotary shaker at room temperature.

Polysulphone membranes (eight pieces of 1 cm^2) and sol-gel matrices were activated with formaldehyde (Merck, Darmstadt, Germany), according to the method described by Dobreva et al. [7], followed by mixing with 5 ml cell suspension, and kept in flasks at 25°C overnight on a rotary shaker. Following the immobilization procedure, abundant rinsing with distilled water was carried out to remove any unattached cells before enzyme activity assays.

Assay methods

Enzyme activity

Enzyme activity was assayed by measuring the ammonia released from benzonitrile according to the method of Fawcett and Scott [9]. One enzyme unit (U) is defined as the amount of enzyme producing 1-μmol ammonia per minute at pH 7.2, 45°C and with 20 mM benzonitrile as a substrate.

Cell concentration

Cell concentration was determined by measuring optical density at 660 nm and calculated as milligram biomass, according to a standard curve with respect to dry weight. One optical unit was established to correspond to 0.6 mg/ml dry cells.

Thermal stability

The free and immobilized cells were incubated in buffer solution (pH 7.2) at 50, 60 and 70°C for different periods of time. At certain intervals the residual enzyme activity of the treated cells was measured.

Operational stability

The batch reusability of the immobilized cells was studied to determine the number of cycles for which it could be used without significant loss of activity. Each reaction cycle was an enzyme reaction for 15 min at 45°C and with 20 mM benzonitrile as a substrate. The enzyme activity was assayed, followed by rinsing with distilled water before the next reaction cycle.

Results and discussion

By measuring the difference in cell concentration between the cells used for immobilization (75 mg, assumed as 100%) and the residual cells found in the rinsing waters, the quantity of immobilized cells was established (Table 1). The efficiency of binding on the

Table 1 Immobilization efficiency according to cell concentration with the different methods used. Percent efficiency calculated relative to total cell concentration of the suspension (75 mg = 100%)

Carrier	Method	Cell concentration		Efficiency (%)
		In the rinsing waters (mg)	Total quantity immobilized (mg)	
Membranes	Binding after activation	64.5	10.5	14
Sol-gels[a]	Entrapment	0	75	100
	Adsorption	67.5	7.5	10
	Binding after activation	66	9.0	12

[a]These results are valid for all types of hybrid sol-gel matrices

Table 2 Immobilization efficiency according to enzyme activity of the different biocatalysts. Percent efficiency is calculated relative to the total activity of cell suspension used for immobilization (11.25 = 100%). *PEG* Polyethylene glycol, *PVA* polyvinyl alcohol

	Enzyme activity of immobilized biocatalyst								
	Entrapped			Adsorbed			Chemically bound after activation		
Carrier	U	%	U/mg cells	U	%	U/mg cells	U	%	U/mg cells
Membranes	–	–	–	–	–	–	5.6	5.0	0.53
PEG	8.3	7.4	0.11	1.0	0.9	0.13	3.3	2.9	0.36
PVA	2.4	2.1	0.03	0.33	0.3	0.04	1.4	1.2	0.15
PEG + PVA	6.1	5.4	0.08	0.8	0.7	0.11	2.5	2.2	0.28

activated membrane surface (14%) is comparatively low; however, all cells used were entrapped in the sol-gel network. In this case leaching is negligible (0.01–0.05%). The lowest quantity of cells (10%) was found on the sol-gel matrix surface at adsorption, but after activation with formaldehyde immobilization efficiency increases by 2%. Cell loading on the biocatalysts was: 1.3 mg/cm^2 on membranes; 18.75 mg/g entrapped in the sol-gel; 3.75 mg/g adsorbed on the non-activated surface and 5 mg/g bound on the activated surface.

The results were different when immobilization efficiency was presented according to the enzyme activity at the beginning and at the end of the immobilization process (Table 2). Nitrilase activity of all immobilized preparations was calculated relative to the total activity of the cell suspension used for immobilization (11.25 U), taken as 100%. For the sol-gel matrices it was 7- to 8-fold higher for entrapped cells than adsorbed cells. When activation was carried out before adsorption, enzyme activity increased 3- to 4-fold. Silica has silanol groups on its surface, which take part in hydrogen bonding as well as electrostatic interactions during adsorption. Additional modification with formaldehyde leads to formation of a stronger chemical bonding between the cell and the modified carrier surface. A similar effect was established in our previous work with polysulphone membranes as cell supports [7]. In the present work the enzyme activity in cells bound on polysulphone membranes was 5% of the total activity, and was higher than that of cells chemically bound on sol-gel carriers (Table 2). Nevertheless, the efficiency of binding is almost the same (Table 1), thus the polysulphone membranes were more suitable for use. The high difference in enzyme activity between cells entrapped and adsorbed on sol-gels was eliminated if activity is calculated as units per milligram of immobilized cells (Table 2). The activity of adsorbed cells then reaches, and even exceeds, the activity of entrapped cells, the reason being different cell loading on the carriers. Although the quantity of adsorbed cells is lower, they are free to react directly with the substrate and no mass transfer limitations exist in the matrix. Immobilized cells after activation of sol-gel carriers showed a 3-fold higher enzyme activity (U/mg cells) than entrapped and adsorbed cells, which is due to the preservation of a constant cell quantity because of the stronger binding. Enzyme activity was highest for cells bound on activated membranes (0.53 U/mg cells).

The influence of the type and quantity of organic material on the immobilization efficiency was found to be of importance (Table 2, Fig. 1). The addition of organic material to the sol-gel had a favorable effect compared to the pure silica matrix. Variation in the amount of PEG, PVA and PEG + PVA from 5 to 20% showed that 5% organic material is the most successful (Fig. 1). Higher concentrations of organic material leads to inhibition of enzyme activity, probably because it becomes toxic to the cell, while low organic concentration makes the sol-gel matrix a more suitable structure for effective cell immobilization. Concerning the type of organic material, the results for enzyme activity were in the order: PEG > PEG + PVA > PVA (3.5:2.5:1.0 for entrapment and 3.1:2.3:1.0 for adsorption), while this correlation in the case of binding after activation was 2.4:2.0:1.0 (Table 2).

The data in Figs. 2 and 3 show the effect of immobilization on the operational stability of immobilized biocatalysts. When sol-gels were used as carriers, stability was lowest for adsorbed cells—the residual activity after five cycles was 20% and for entrapment, 40%, respectively. This fact is explained by the more feeble bonds at adsorption; immobilized cells were easily washed out. Activation of the carriers with formaldehyde considerably increased the operational stability of the biocata-

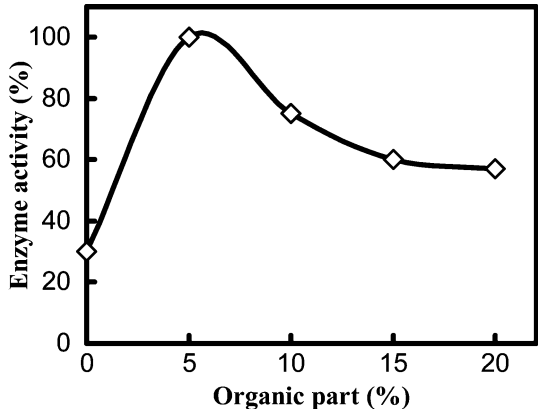

Fig. 1 Influence of the concentration of organic material in the matrix on enzyme activity of immobilized cells

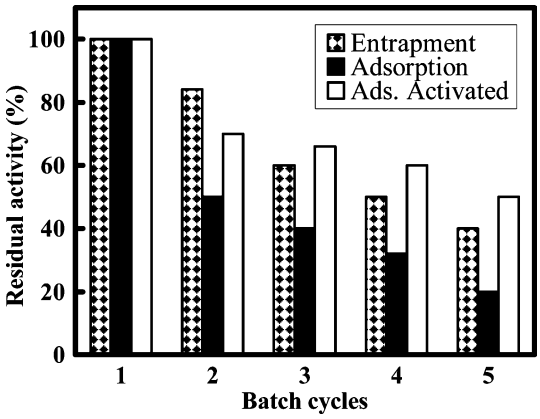

Fig. 2 Residual activity of cells immobilized on sol-gel matrices after repeated use for benzonitrile degradation

lysts because it leads to the formation of stronger bonds. In this case, the residual activity after five cycles was 50%. The polysulphone membranes gave the best results for operational stability (Fig. 3). Regarding batch reusability of membrane-immobilized cells, 100% enzyme activity was retained after eight reaction cycles. In the ensuing cycles a gradual decrease was encountered, with a residual activity of 50% after 10 cycles.

The effect of high temperatures on the activity of free and immobilized cells (on activated hybrid silica matrices with 5% PEG and on polysulphone membranes), showed that thermal stability in both types of immobilized cells was better than that of free cells (Fig. 4).Thermal stability was not studied at lower temperatures because free cells were stable enough for more than 2 h. At 60°C the half-lives of activity were 27 min for free cells, 38 min in the case of sol-gel carriers and 50 min for membrane-immobilized preparations (Fig. 4a). At 70°C the results were in the same order (Fig. 4b); half-lives were 4.5, 6 and 11 min, respectively.

The use of biological systems to convert nitrile-containing substrates to carboxylic acids is an attractive alternative to chemical methods. A bioremediation process could be conducted efficiently using immobilized cells with nitrilase activity. The new sol-gel hybrid

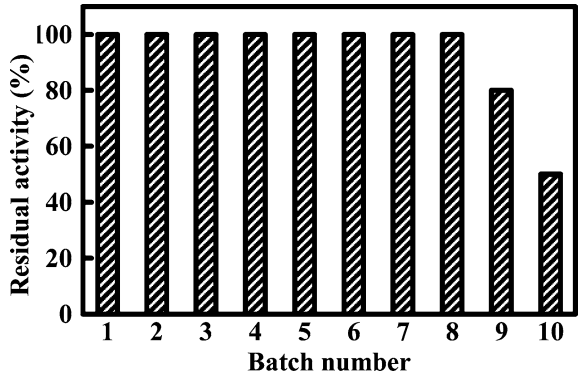

Fig. 3 Residual activity of cells immobilized on polysulphone membranes after repeated use for benzonitrile degradation

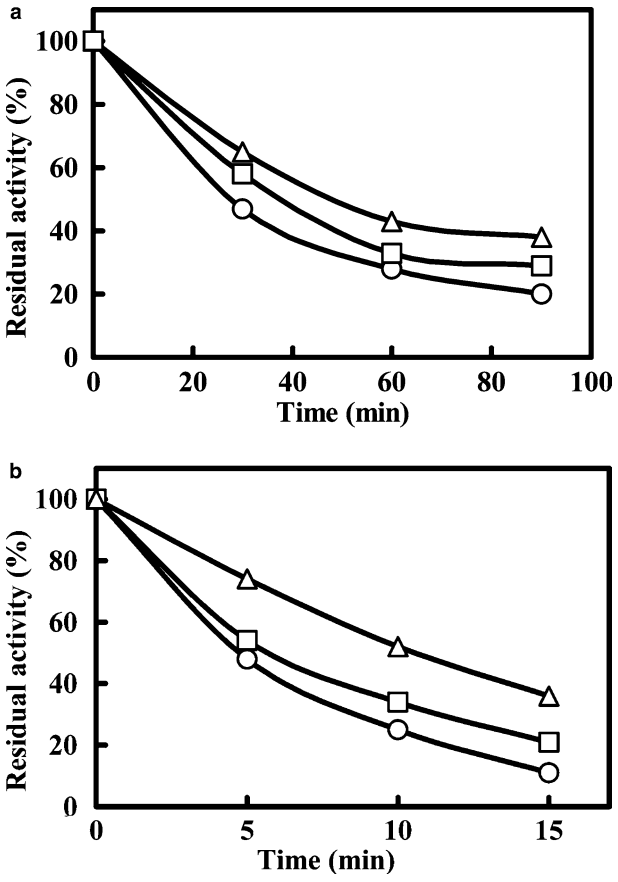

Fig. 4 Thermal stability at 60°C (**a**) and 70°C (**b**). *Circles* Free cells, *squares* cells immobilized on sol-gel matrices with 5% PEG, *triangles* cells immobilized on polysulphone membranes

materials appear to be suitable matrices for immobilization of whole bacterial cells of *Bacillus* sp. UG-5B strain for nitrile degradation. The living cells were trapped directly within the sol-gel matrix or on the carrier surface but they retain their bioactivity and are accessible for reaction with the substrate.

Among the supports screened, membranes gave best results in relation to immobilization efficiency and operational stability. Future scaling up to continuous bioconversion in column reactors is feasible.

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