

Silica-carrageenan hybrids used for cell immobilization realizing high-temperature degradation of nitrile substrates

Research Article

Lyudmila V. Kabaivanova¹, Georgi E. Chernev^{2*},
Isabel M. Miranda Salvado³, Maria H.V. Fernandes³

¹Institute of Microbiology, Bulgarian Academy of Science,
1400 Sofia, Bulgaria

²Department of Silicate Technology, University of Chemical Technology
and Metallurgy, 1756 Sofia, Bulgaria

³Department of Ceramic and Glass Technology, University of Aveiro,
CICECO, 3810-193 Aveiro, Portugal

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Abstract: In this work the application of hybrid materials, containing TEOS as source of SiO₂ and κ-carrageenan in different percentage, synthesized by the sol-gel method at room temperature was studied. They were used as matrices for entrapment of whole *Bacillus sp.* UG-5B cells, producers of thermostable nitrilase. The effect of the surface area and size and quantity of pores in the synthesized materials on the enzyme activity was evaluated. The process of biodegradation of different concentrations of toxic, potentially carcinogenic and mutagenic substrates by the obtained biocatalysts was investigated. The enzyme reaction takes place by the nitrilase pathway, catalysing nitrile hydrolysis directly to the corresponding carboxylic acid, forming ammonia. At batch experiments the influence of the substrate concentration of different nitriles was tested and 20 mM concentration was found most suitable. A two-step biodegradation process in a laboratory-scale column bioreactor of o-, m- and p-tolunitrile as a mixture was followed. After operation of the system for nine hours for the mixture of substrates at a flow rate of 45 mL h⁻¹ and at 60°C, the overall conversion realized was above 90%, showing a good efficiency of the investigated process.

Keywords: Sol-gel • Carrageenan • Entrapment • Biodegradation • Cyanides

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1. Introduction

Cell immobilization technology consists in the attachment of cells to a certain separated insoluble phase different from the phase of the free solution but capable of exchanging substrate and product molecules and accomplishing of the enzyme substrate reactions [1-3]. Immobilization methods are mainly chemical and physical. Chemical methods involve covalent bond formation between the surface of the cell and carrier, while the physical ones are characterized with electrostatic interactions, ionic bonds, hydrophobic influences [4,5]. According to the purpose of the experiments, adsorption, chemical binding after activation or entrapment inside the volume of the carrier can be chosen as to realize an effective and low cost process. Different carriers have

been tested for immobilization of various types of living cells to achieve utilization of biochemical functions of active biomolecules, giving good yield of the developed processes [6,7].

Creating biocatalysts by immobilized whole microbial cells finds its application in a wide spectrum of biotechnological processes, starting from the ethanol production [8] to phenol degradation [9,10]. Immobilized cell bioreactors have been used due to their stability and possibility to achieve a continuous biodegradation process [11].

Using different biocatalysts is expected to reduce the energy expenses and their employment could decrease the distribution of various pollutants in the environment [11-13]. The wastewater treatment technologies could be significantly developed by the introduction of new

* E-mail: georgi_chernev@yahoo.com

microbial treatment [14,15]. One part of this problem is the detoxification of contaminated hot industrial waters before cooling. The application of biological systems for conversion of nitrile compounds to the corresponding acid and ammonia is an attractive alternative to the chemical methods due to the good yield, the mild reaction conditions and the specific activities of the enzyme systems [16].

Nitriles are toxic, carcinogenic and mutagenic organocyanides, which are often found in the environment as a result of different industries and may persist in soil or water for a long period and cause harmful disturbances to human health. There exists an urgent need for development of procedures that reduce the nitrile concentrations throughout the environment in that way reducing the impact of nitrile wastes streams. Immobilization of nitrile-converting bacteria as means of increasing catalyst longevity in chemical synthesis [17] or for their use in detoxification of contaminated sites has been of particular interest lately [18].

Combining silicon chemistry with life sciences makes possible the application of hybrid biomaterials as carriers for different biomolecules, including cells [18,19]. The preparation of bioactive composites could be achieved by the developments in sol-gel techniques [20,21]. For successful implementation of this task the biological reagent should remain intact within the suitable hybrid material with high activity and long-term stability, good resistance to leaching and desorption [22]. Sol-gel materials are promising for application since they have demonstrated to be highly compatible with enzymes and cells even stabilizing and protecting them with their great mechanical, thermal and biological resistance [23,24]. A new field in the sol-gel technology is the synthesis of hybrid materials, simultaneously containing inorganic and organic components. Their physical and

chemical properties can be controlled by variations in the quantities of the constituents and depend on the synergism between them [25]. It is supposed that interaction between phases in the hybrid materials, where the inorganic phase is often covalently bonded with the organic polymer, will lead to more valuable properties compared to classical composites [26].

The aim of the present study was sol-gel synthesis of hybrid matrices containing k-carrageenan for obtaining stable and effective biocatalysts for enzyme biodegradation processes of different toxic substances at high temperature.

2. Experimental Procedure

2.1. Microorganism and culture conditions

Cells of the moderate thermophilic strain *Bacillus* sp. UG-5B with nitrilase activity, deposited in National Bank of Industrial Microorganisms and Cell Cultures-Bulgaria #8021/2001, isolated from polluted industrial waters near the refinery "Neftochim" Bourgas, were used in the immobilization procedures. Cultivation was carried out at 60°C. The medium composition was (g L⁻¹): Reptone-5.00 (Oxoid); Yeast extract-5.00 (Oxoid); K₂HPO₄-5.00; MgSO₄-0.10; NaCl-1.00; FeSO₄·7H₂O-0.03; benzonitrile -20 mM, pH-7.5. *Bacillus* sp. UG-5B was grown in Erlenmeyer flasks (500 mL) for 14 h. The shake-flask culture was grown in a rotary shaker (Gyrotory water bath shaker New Brunswick Scientific). Separation of the cells from the culture medium was performed at the beginning of stationary state by centrifugation and after that they are re-suspended in a phosphate buffer solution (0.06 M, pH 7.2). Cell suspension with a concentration of 35 mg mL⁻¹ dry cells and nitrilase activity of 2.8 U mL⁻¹ was used for entrapment.

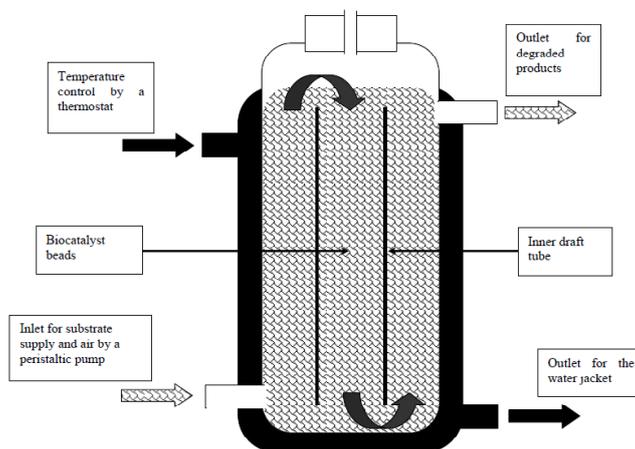


Figure 1. Schematic presentation of laboratory scale bioreactor used for biodegradation of the mixture of cyanide compounds

2.2 Chemicals

The silicon alkoxide precursor: tetraethylortosilicate (TEOS) purchased by “Merck” was used. H_2O ; 0.1 N HCl and phosphate buffer with $pH=7.2 \pm 0.02$ at $20^\circ C$ (0.06 M) -“Merck” were also used in the synthesis. The organic part involved was k-carrageenan (Merck) in quantity 5 wt% (solubility 0.5 g in 100 mL hot water, $20^\circ C$). Benzonitrile and tolunitriles, used as substrates in the enzyme reactions were purchased by “Fluka”.

2.3. Matrix synthesis

Sol-gel transparent silica hybrid matrix with 5, 10 and 20 wt% organic component was synthesized at room temperature and controlled pH conditions. A poly-step sol-gel procedure was carried out at strictly controlled conditions in order to obtain the desired structured materials. No alcohol was added as a co-solvent. 5 mL of 0.1 N HCl were introduced to reach $pH \sim 1.5$ of the initial solution for increasing hydrolysis rate. The inorganic-organic hybrid materials were prepared by substituting part of the inorganic precursor with k-carrageenan. The pH was raised up to $pH=7.2 \pm 0.02$ at $20^\circ C$ with phosphate buffer to keep cell vitality. The quantity of buffer solution varies for the different sol mixtures depending on the organic component content from 15 to 20 mL to increase pH to 7.0 of the final mixture before introduction of the cell suspension. Before addition we shake the cell suspension well, after which, the whole solution including cell suspension and sol mixture is slightly stirred to obtain a homogeneous mixture. In all cases the ratio precursor/ H_2O was kept constant and equal to 1. No phase separation was observed before and after the gelation point. The addition of organic component leads to a faster polymerization. Thin transparent hybrid flakes were obtained in the hybrid synthesis. The drying procedure was carried out at room temperature overnight.

2.4. Immobilization and bioreactor setup

Sol-gel hybrids containing 5 wt% k-carrageenan were prepared as flakes with the inclusion of 10 mL of the cell suspension. The living cells were trapped directly within the gel matrix before gelation. The obtained biocatalysts were applied for packing of the laboratory column bioreactor (Fig. 1). It possesses a working volume of 100 mL and glass walls, forming a cylinder. After packing with the biocatalyst the temperature was controlled and maintained at $60^\circ C$ by circulating water through the water jacket connected to a thermostatic bath. The reactor contained 45 g of the ready biocatalyst. The substrate solution (20 mM mixture of o-, m- and p-tolunitrile) was fed into the bioreactor using a peristaltic pump, which

regulated the flow-rate. The bioreactor performances were carried out at a flow rate of 45 mL h^{-1} .

2.5. Assay methods

Optical density was measured at 660 nm to estimate cell quantity in the rinsing waters and leakage of cells. Enzyme activity was assayed by measurement of ammonia released due to nitrilase action according to the phenol-hypochloride method of Fawcett and Scott [27]. One enzyme unit (U) is defined as the amount of enzyme, producing $1 \mu\text{mol ammonia min}^{-1}$ at pH 7.2, $45^\circ C$ and 20 mM benzonitrile as a substrate. All runs were replicated and averaged values are reported.

2.6. Matrix investigations

For studying the structure of the synthesized hybrids the following methods were used: FT-IR (IR- MATSON 7000-FTIR), XRD (X-ray PW1730/10, the diffracted intensity of Cu $K\alpha$ radiation was measured with scan rate of $0.02^\circ \text{ min}^{-1}$ in 2θ range between 4° and 80°), BET-Analysis (Gemini 2370 V5), Energy Dispersive System (EDS) (RONTEC EDS), SEM (Philips-515), AFM (NanoScope Tapping ModeTM) and roughness analysis. The influence of the hybrid matrix, synthesized with TEOS and k-carrageenan on the cells of the investigated strain was evaluated by scanning electron microscopy (Scanning device attached to Zeiss electron microscope-model 10C, at 20 kV accelerating voltage with electron beam 5-6 nm) on the surface of a piece of this type of matrix.

3. Results and Discussion

The FT-IR spectra of synthesized inorganic-organic materials show the bands at 1080 cm^{-1} , 790 cm^{-1} and 480 cm^{-1} . They are assigned to ν_{as} , ν_s and δ of Si-O-Si vibrations, but at the same time these bands can be related to the presence of Si-O-C, C-O-C and Si-C bonds. The band at 960 cm^{-1} is due to a stretching Si-OH vibration. The bands at 1439 cm^{-1} are assigned to C-O-H vibrations. The characteristic bands at around 3450 cm^{-1} and at 1640 cm^{-1} assigned to H-O-H vibration can also be found (Fig. 2). It can be seen that the transmittance of hybrids decreases with increasing of carrageenan content.

The results from the XRD-analysis show that all the studied hybrids were in an amorphous state. It was established that the diffraction peaks intensity decreased with an increase of organic component content. At the same time the type of the XRD patterns indicates that some processes of ordering were carried out.

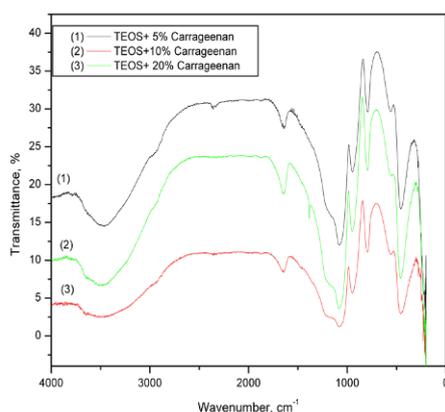


Figure 2. FT-IR spectra of synthesized hybrid materials containing TEOS and different percent k-carrageenan

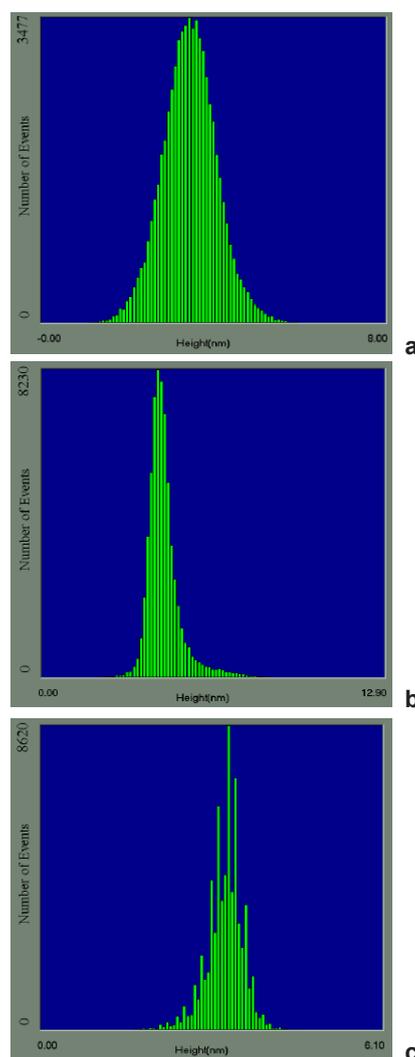
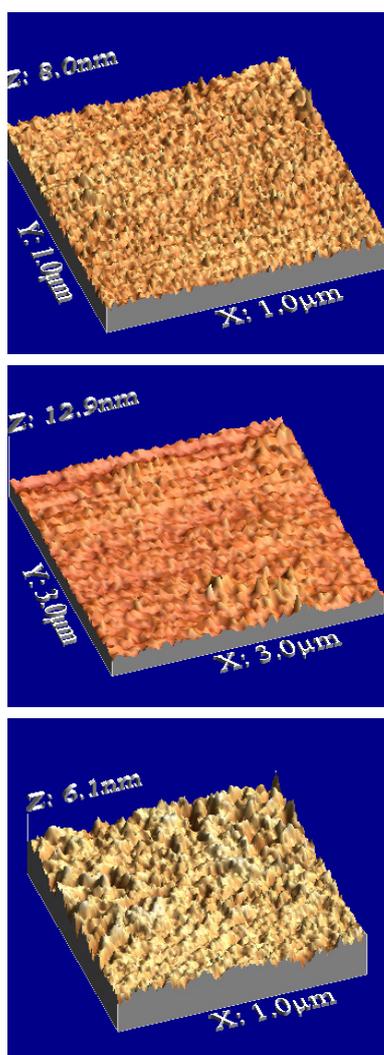


Figure 3. AFM and roughness analysis of synthesized hybrid materials containing TEOS and different percent k-carrageenan (a) 5 wt%; (b) 10 wt% and (c) 20 wt%

The chemical content in the samples determined by EDS showed presence of Si, O, N, Na, P, S, Cl and K. From the data of BET analysis it was established that the surface area is in the range of 396 to 260 m² g⁻¹. The results clearly show that with increasing the percent of the organic component, the surface area decreases.

From the AFM analysis a self-organized nanostructure was established (Fig. 3). All synthesized hybrid samples have surfaces with irregularities of quite small height. In the obtained hybrids the nanobuilding blocks are made up of nanounits of SiO_x groups and that van der Waals and Hydrogen bonding or electrostatic interactions between the nano-building blocks exist. In the synthesized samples the average size of particles is about 8 - 12 nm and the dimensions of their aggregates are about 16 - 49 nm. The surface roughness of

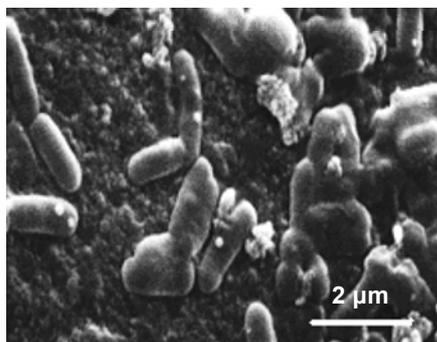


Figure 4. Image taken by scanning electron microscopy of the cells on a piece of hybrid matrix with k-carrageenan

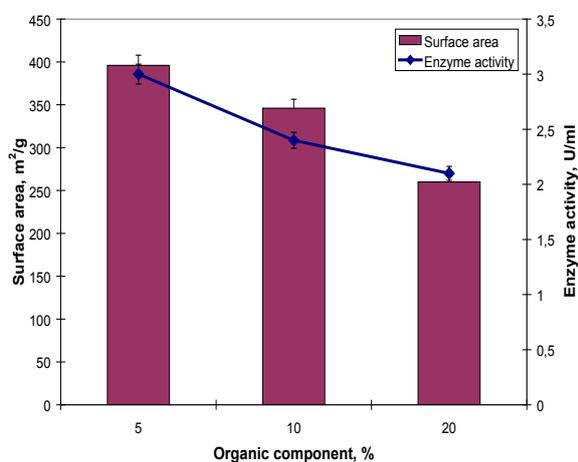


Figure 5. Correlation between surface area and enzyme activity using three types of matrices – with 5, 10 and 20 wt% organic constituent

the synthesized hybrids depends on the quantity of k-carrageenan. With increasing of its percentage, the surface roughness increases.

The obtained hybrid material appeared to be compatible with cells. Water solutions of silica precursor with the purpose to reduce the quantity of alcohol in the primary mixture were used. The time for homogenization before the addition of organic part and cell suspension was increased in order part of the alcohol formed during hydrolysis to be evaporated. Addition of phosphate buffer for raising the pH of initial sol in a way decreases the exposure of cells to acid and high alcohol concentration [28]. The remaining quantity of alcohol did not appear deleterious to the cells as the enzyme systems responsible for nitrilase synthesis function. All immobilized cells showed nitrile-degrading activity immediately after the preparation and lasting for a long period of time. Immobilized cells can be re-used in batch cycles up to 20 each with fresh substrate medium. Their cellular organization and enzymatic activity appeared to be preserved after encapsulation. Bacteria that are damaged or even dead may still maintain some

enzymatic activity and could then behave just as a “bag of enzymes” [29].

Our previous investigations showed that the type of matrix influences to a certain extent the activity reached, together with other factors that have been changed for optimization of the process. We have used different type of organic constituents: agar, PAAG, PEO, alginate, chitosan and chitin in different percentage [30–33].

Hydrolysing activity of nitrilase from *Bacillus sp.* UG-5B in batch experiments was established towards different concentration of nitriles for free and immobilized cells (Table 1). The enzyme activity towards benzonitrile was assumed as 100%. For 20 mM concentration, 100% corresponded to 2.8 U mL⁻¹; for 30 mM concentration – 2.2 U mL⁻¹ and for 40 mM concentration - 2.1 U mL⁻¹. In this study the relative enzyme activity of free and entrapped cells was compared for o-, m- and p-tolunitrile, representatives of the carboxylic nitriles. From the group of heterocyclic nitriles, 4-cyanopyridine was tested to find that with this type of matrix the values of the relative nitrilase activity for entrapped cells did not exceed these for the free cells. This is the reason why 4-cyanopyridine was not subjected to degradation in the reactor with the immobilized biocatalyst. The catalytic route of nitrile transformations is of particular interest because the nitrile-degrading enzymes can convert diverse nitrile substrates into various amides and carboxylic acids under relatively moderate process conditions with excellent chemo-, regio- and stereoselectivities. An important development in the area of nitrile and amide hydrolytic bioprocesses has been the use of immobilized biocatalysts, which confers the benefits of biocatalyst retention and reuse in continuous process strategies [34–36]. As it can be seen from Table 1 highest enzyme activity was shown towards p-tolunitrile, probably due to a stereo-selectivity, characteristic for the nitrile – converting microbial enzymes [37] and the type of the synthesized matrix, so o- and m- tolunitriles are less susceptible to hydrolysis for both free and immobilized cells. The final product of enzyme reaction was only benzoic acid detected by gas chromatography analyses. Benzamide was not found as an intermediate product. The influence of the concentration of the substrate was also shown, confirming that concentration of 20 mM was most appropriate to carry out the enzyme-substrate reaction. It is evident that the increase of the substrate concentration up to 40 mM did not lead to increase in the nitrilase activity. Higher concentrations of these toxic substrates probably affect negatively the cells and their enzyme systems.

The scanning electron micrograph represents part of the matrix where undamaged cells can be clearly seen to prove that they keep their integrity and no lysis was

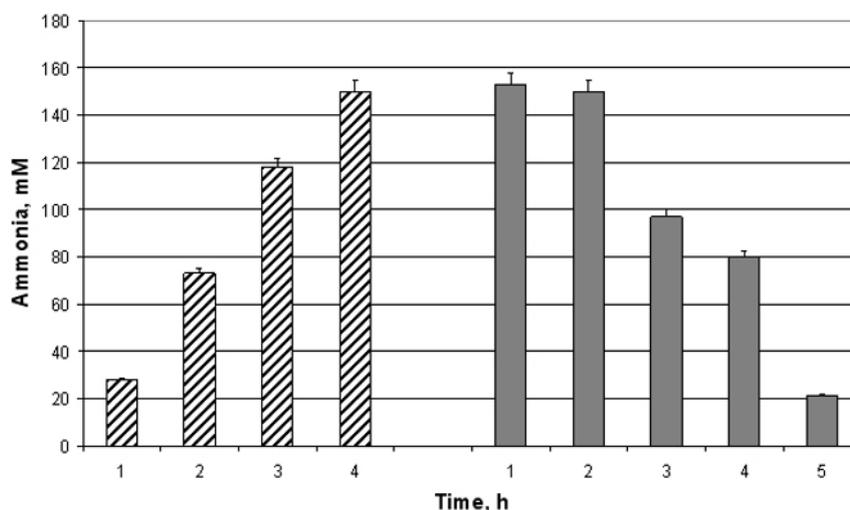


Figure 6. Quantity of ammonia released, corresponding to the quantity of degraded substrate-mixture of tolunitriles in the bioreactor; ▨- first step of the process; ■- second step

Table 1. Influence of the substrate concentration on the relative enzyme activity at batch experiments.

Type of substrate	Substrate concentration, mM	Relative enzyme activity of free cells, %	Relative enzyme activity of entrapped cells, %
o-tolunitrile	20	14	39
	30	8	30
	40	14	10
m-tolunitrile	20	30	48
	30	20	36
	40	19	12
p-tolunitrile	20	80	95
	30	30	80
	40	28	80
benzonitrile	20	100	100
	30	100	100
	40	100	100

observed (Fig. 4). We know from literature data that the sol-gel process allows the encapsulation of living cells due to its features, such as low temperature processing, high stability, pH regulation and contact surface area, which facilitates permanent interactions between the microorganisms and the substrate, and does not prevent cell reproduction [38].

The influence of the surface area and the size of pores on the enzyme activity of entrapped cells were also studied (Fig. 5). The obtained results showed that the surface area of the samples with 5% k-carrageenan is the biggest, reaching $396 \text{ m}^2 \text{ g}^{-1}$ and the pore size is 1.6 nm which promotes highest enzyme activity in comparison to the sample with 20% k-carrageenan where the surface area is $260 \text{ m}^2 \text{ g}^{-1}$ and the pore size is 2.3 nm. In the sample with 5 wt% organic part the pore size is the least, but enough for the substrate and oxygen to penetrate.

The next step of the investigation was to prepare a laboratory-scale column bioreactor filled with 45 g of

hybrid material with 5 wt% carrageenan, containing the entrapped bacterial cells. After entrapment, the whole cell quantity used (350 mg) was found within the gel. Leaching was 0.003%.

The process of enzyme biodegradation of the three substrates: o-; m- and p-tolunitrile in two steps at 60°C were followed. The remaining quantities of not degraded substrates at the first step were treated for a second time in the reactor filled with the already released solution after the first step for another five hours for the mixture of tolunitriles.

In nature the pollutants often occur as mixtures so o-; m- and p-tolunitrile were treated as a model system in the bioreactor. To realize the biodegradation of this mixture of nitriles nine hours were needed to obtain almost full substrate degradation. At the first step (four hours) a degradation of 150 mM was achieved. After the fifth hour a depletion of the substrate was established (Fig. 6) to realize a bioconversion of the model system of tolunitriles up to 94.9 % for nine hours.

To obtain biocatalysts with broad substrate specificity, increased operational stability and thermostability by applying new carriers for effective immobilization and new methods for biotransformation of complex molecules, becomes the aim of the investigations of many researchers. Using biocatalytic processes, nitrile substrates can be readily hydrolyzed under moderate conditions, compared with the relatively severe conditions required for analogous chemocatalytic processes [39,40].

Our results confirm the statement that microbial cells could be involved by immobilization in processes of remediation of contaminated sites, particularly when polluted industrial waters come out hot and can be easily treated immediately without cooling for a short period, since with the increase in temperature the reaction rate increases while energy requirements decrease. The biocatalysts with immobilized cells possess the advantage of carrying out an economic process of continuous use at stable conditions and control over the specific enzyme reactions. The technique of immobilization in sol-gel matrices has proved to be biocompatible and easy to perform [41]. Preservation of microorganisms is an important feature for biotechnology [42]. Together with viability and activity of the enzyme, a long-term maintenance is required. Here is the role of immobilization. The porous structure of the hybrids is a major parameter for the efficiency of the immobilization process as the pores of the hybrid materials permit the diffusion of the substrates and products of the enzyme reaction.

Certain differences were established between free and entrapped cells at the batch experiments, using different nitriles which fact can be devoted to the formation of a microenvironment around the cell at entrapment. This is valid in the cases when the enzyme activity is higher for entrapped than for the free cells. The same fact was proved by Graham [23] who stated the immobilization inside a gel lead to additional stabilization of the cells towards high concentration of organocyanide substrates. For the remaining biocatalysts where the enzyme activity of free cells is higher it could be suggested that the formation of a barrier may serve as a hindrance for the penetration of the substrate inside the cells.

Since one of the fastest growing research directions in the field of sol-gel science and technology is the immobilization of different living cells in sol-gel matrices

retaining their bioactivity, we tried to prove this feature. The obtained biocatalysts on the basis of silica sol-gel hybrids with 5 wt% k-carrageenan proved to be suitable for this purpose.

4. Conclusions

A poly-step sol-gel procedure was used at strictly controlled conditions different from the conventionally used, concerning the solvents and acidity in order to keep cell vitality and to obtain the desired hybrid materials. The introduction of an organic constituent to produce a hybrid material showed its favourable effect on the biocatalyst formed. Introduction of 5 wt% of this organic substance is sufficient and promoting higher enzyme activity reached 3.0 U mL^{-1} , compared to the this obtained when the cells were immobilized in hybrid matrices containing 10 wt% (2.4 U mL^{-1}) and 20 wt% organic part (2.1 U mL^{-1}).

The accomplishment of a biodegradation process in a bioreactor proved no washout, allowing high and stable process efficiency even under variations in toxicity, concentration and at high temperature. In general, the diffusion of organic pollutants correlates to the solubility, which increases with temperature in two steps for 9 hours to reach degradation of 94.9% of the nitrile mixture. An increase in the reaction temperature had its favourable effect on the bioreactor efficiency, probably due to increased mass transfer. The bacterial cells immobilized in the hybrid materials have shown preservation of the enzyme systems, responsible for the nitrilase synthesis. In this study the high efficiency of nitrile degradation by immobilized bacterial cells showed a high potential as a bioremediation technique for the treatment of organocyanides as the strain *Bacillus* sp. UG-5B has shown a broad substrate specificity, hydrolysing aliphatic, carbocyclic and heterocyclic nitriles. The problem about the biological treatment of hot waste waters before cooling has always been of importance. Immobilization of such thermophilic cells in sol-gel hybrid matrices permits, performing of the process of biodegradation at high temperature with high speed and least contamination. The possibility to carry out this particular biotransformation in a series of reactors with immobilized biocatalysts at high temperature, reaching almost full bioconversion values is feasible.

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