

## Hybrid materials parameters influencing the enzyme activity of immobilized cells

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Hybrid materials, synthesized by the sol-gel method, were used as matrices for immobilization of bacterial cells, producers of the enzyme nitrilase. Different methods were employed for structure investigations of the synthesized hybrids: Fourier transform infrared spectroscopy (FT-IR), BET and atomic force microscopy (AFM). The influence of the structure of these materials on their properties was followed. The obtained hybrid materials were successfully applied for immobilization of live bacterial cells since cell vitality was kept and enzyme systems preserved their functions in the immobilized systems. The influence of different parameters during the cell immobilization procedure was evaluated for optimization of the immobilization process and enhancement of the activity of the studied enzyme. Most favorable effect on *Bacillus sp.* cells had the matrix with 5 wt. % sepharose and the enzyme activity obtained was 0.45 U ml<sup>-1</sup> when the process was carried out at 60°C. Increased specific surface area of the matrix was another factor that led to enhanced nitrilase activity.

**Keywords:** sol-gel; hybrids; parameters; enzyme activity

### INTRODUCTION

Inorganic-organic hybrids, synthesized by the sol-gel method, are a remarkable group of amorphous nanocomposites, investigated for application in biotechnology, medicine and other areas due to their mechanical, optical, structural and thermal properties, as a result of combining silicon chemistry with life sciences [1-2]. Such hybrid materials are promising for applications as biocatalysts by immobilization of different biomolecules [3-4]. These biocatalysts are expected to reduce the energy expenses and their employment could lower pollutants dissemination in the environment [5-6]. Nitrilases (EC.3.5.5.1) are the enzymes that convert nitriles to the corresponding acid and ammonia. Nitrilases are thiol enzymes that perform an array of carbon-nitrogen bond hydrolysis and condensation reactions [7]. Enzymes degrading nitriles can be found in many microorganisms [8]. Their high chemical specificity makes them attractive versatile biocatalysts [9]. Nitrile-converting enzymes are becoming commonplace in the synthesis of pharmaceuticals and commodity chemicals,

showing their potential in the synthetic biocatalysis, as well as in bioremediation processes, degrading a broad spectrum of toxic substrates [10].

In this work we report on the influence of temperature, type and quantity of organic material included in the matrix and specific surface area of the hybrid materials on the enzyme activity during the immobilization of the moderate thermophilic strain *Bacillus sp.* UG-5B.

### EXPERIMENTAL

#### *Hybrid materials preparation and characterization*

The silica hybrids were prepared by the sol-gel method at room temperature. Silica precursor tetraethylortosilicate (TEOS) (Sigma-Aldrich, reagent grade ≥98%, formula weight 208.33 g/mol) was pre-hydrolyzed with H<sub>2</sub>O using a small quantity of hydrochloric acid as a catalyst. The molar ratio between TEOS:H<sub>2</sub>O:C<sub>2</sub>H<sub>5</sub>OH:HCl was (v/v) 1:2:8:1×10<sup>-3</sup>. The content of silica was kept constant and hybrids containing from 5 to 40 wt.% organic part (lactic acid (Sigma-Aldrich, natural ≥85%, FG, formula weight 90.08 g/mol) or sepharose 4B (Sigma-Aldrich, exclusion limit 60,000 for proteins, 30,000 for dextrans) and combination of poly(ethylene oxide) (Sigma-

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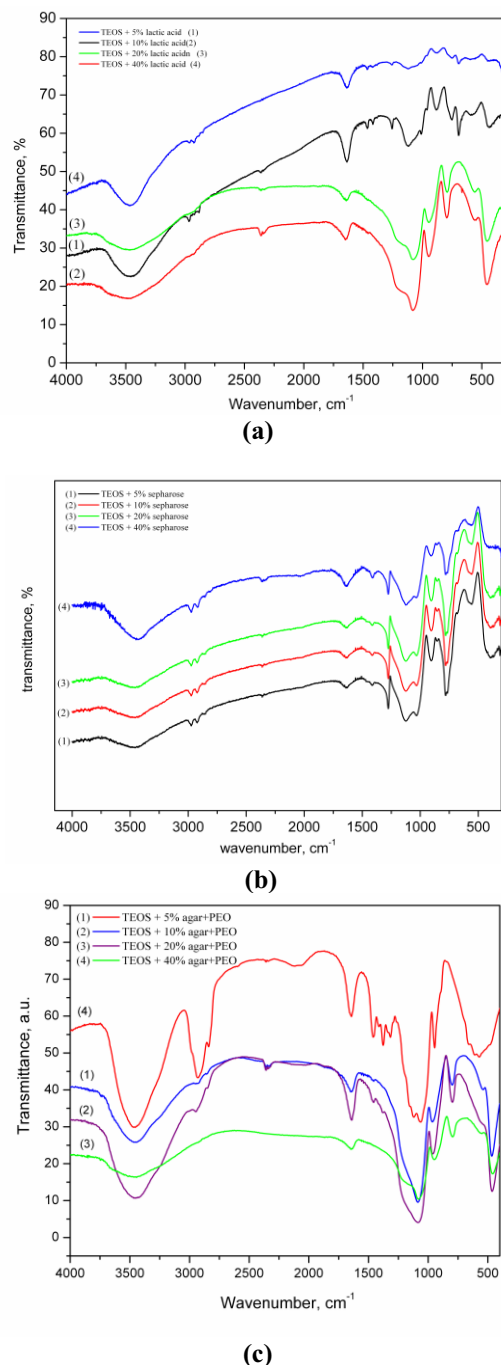
Aldrich, average  $M_v$  100,000, viscosity 12-50 cP, 5 % in  $H_2O$  (25 °C, Brookfield) (lit.)) and agar (Sigma-Aldrich, Type A)) added to  $SiO_2$  were prepared. After gelation, the samples were dried at room temperature. The main steps of the entrapment procedure were previously described [11]. The structure of the synthesized materials was studied using Fourier transform infrared spectroscopy (FT-IR), BET and atomic force microscopy (AFM).

#### Strain and medium

The bacterial strain involved was *Bacillus sp.* UG-5B (№ 8021) deposited in the National Bank for Industrial Microorganisms and Cell Cultures, Bulgaria. The medium for cultivation contained (g/l):  $K_2HPO_4$  2.0; NaCl 1.0;  $MgSO_4$  0.01;  $FeSO_4 \times 7H_2O$  0.02; biotin  $2.9 \times 10^{-5}$ ; thiamine, 0.004; inositol 0.002 and benzonitrile (20 mM), pH 7.2. The nitrilase activity assay was realized by measuring the ammonia released, following the phenol-hypochloride method of Fawcett and Scott [12]. One enzyme unit is defined as the amount of enzyme producing 1  $\mu mol$  ammonia  $min^{-1}$  at pH 7.2, 45 °C and 20 mM benzonitrile as a substrate. Cells were harvested for 24 h on a rotary shaker in 100 ml flasks with nutrient medium containing 20 mM benzonitrile. Cells were separated from the culture medium by centrifugation and then resuspended in phosphate buffer pH 7.2. Cell suspension with a concentration of 30  $mg\ ml^{-1}$  dry cells and enzyme activity of 0.3  $U\ ml^{-1}$  was used for immobilization. Immobilization procedure comprises the preparation of the gel solution which contains  $SiO_2$  and organic parts in a ratio from 5 to 40 wt.%; 5 ml of the ready cell suspension was introduced before gelation, which took place within 30 min. The gel containing the cells was spread over petri dishes and left to dry overnight at room temperature. Leaching of cells was spectrophotometrically estimated to be 0.02-0.06 %.

#### RESULTS AND DISCUSSION

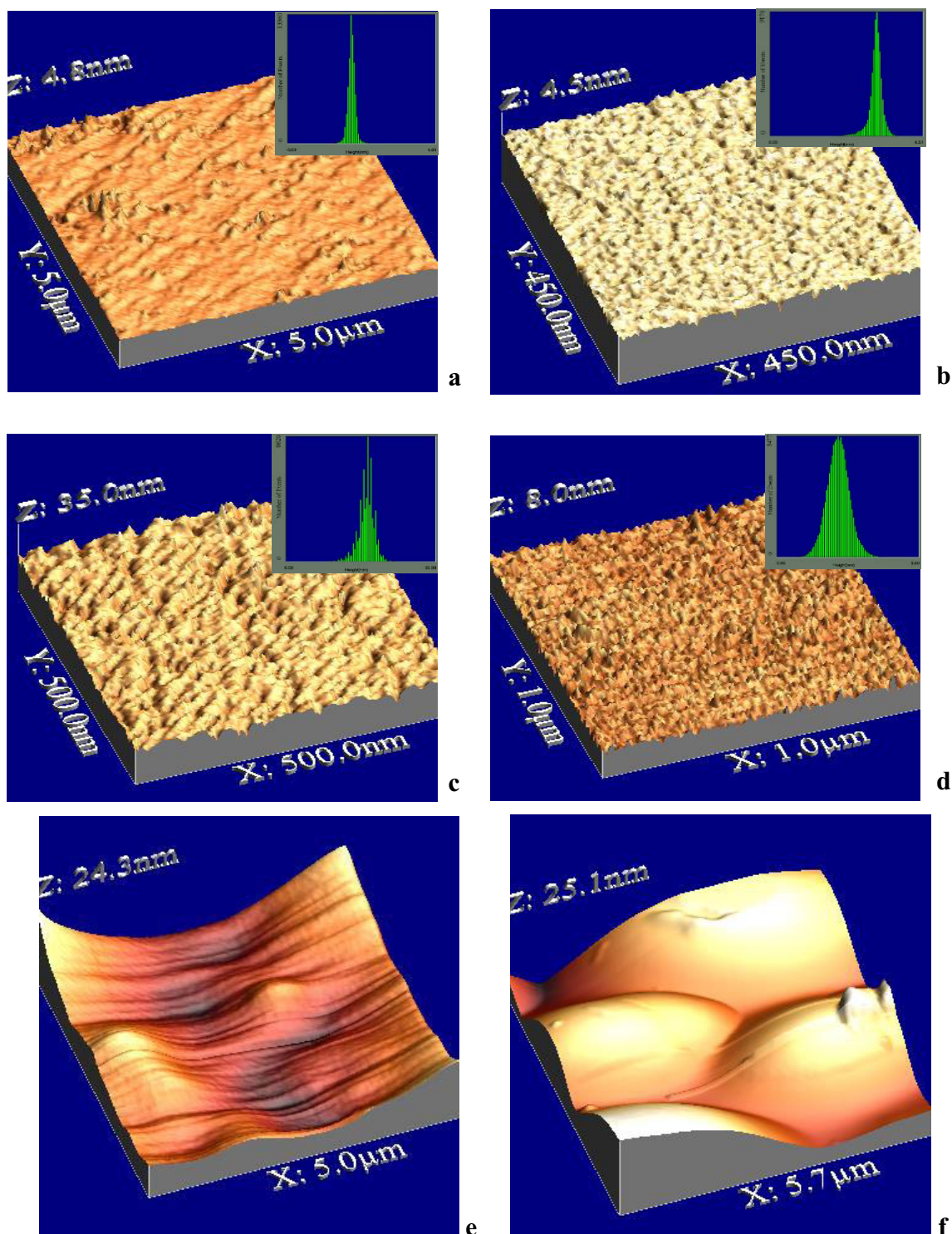
The structure of the synthesized hybrid materials was investigated using FT-IR (Fig. 1), which showed characteristic peaks at around 1080  $cm^{-1}$ , 790  $cm^{-1}$  and 460  $cm^{-1}$ , attributed to the  $SiO_2$  network. They are assigned to  $\nu_{as}$ ,  $\nu_s$  and  $\delta$  of Si-O-Si vibrations; the band at 1080  $cm^{-1}$  could also be related to the existence of Si-O-C, C-O-C and Si-C bonds. The presence of bands at 950  $cm^{-1}$  reveals



**Fig. 1.** FT-IR spectra of hybrids containing: a - lactic acid; b - sepharose and c - PEO and agar.

the presence of Si-OH groups. Characteristic bands at around 3460  $cm^{-1}$  and at 1630  $cm^{-1}$ , assigned to H-O-H vibration, can also be detected. The bands around 3460  $cm^{-1}$  could also be associated with  $\nu$  (OH) ring stretching vibrations.

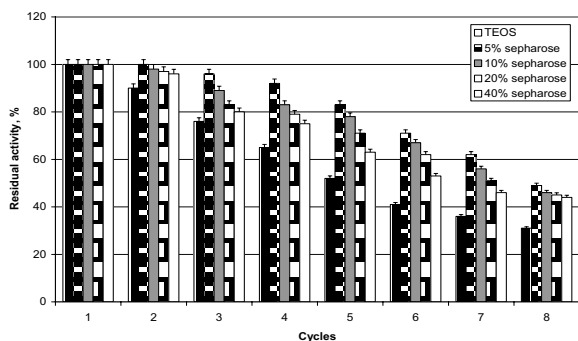
The results of BET analysis proved that the pore size is about 2 nm, and the specific surface area is in the range from 130 to 310  $m^2/g$ , depending on the hybrid chemical composition. The matrix



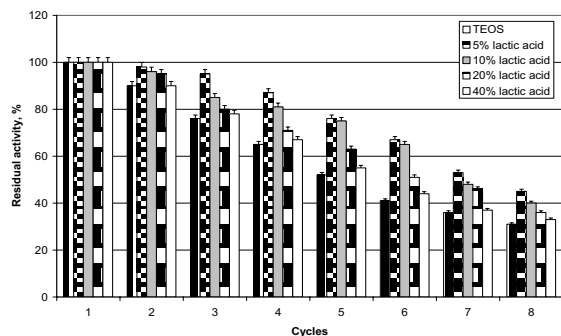
**Fig. 2.** AFM images and height distribution profile of hybrids containing a – 5 wt. % lactic acid; b – 20 wt. % lactic acid; c – 5 wt. % sepharose and d – 20 wt. % sepharose; e – 5 wt. % PEO and agar and f – 20 wt. % PEO and agar.

consisting of well-defined nanounits and their aggregates formed by self-organizing processes, was observed by the AFM studies. The size of nanoparticles was from 10 to 18 nm and the dimensions of their self-assembled aggregates were about 49-76 nm (Fig. 2). In the same figure the height distribution profiles of surface roughness are shown. The histograms of the surface height

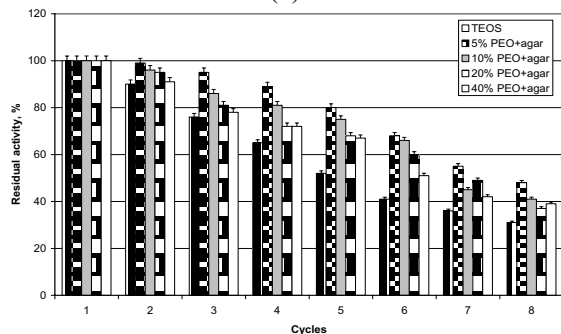
distribution profiles obtained from AFM topography images showed that all hybrids had a surface with irregularities.



(a)

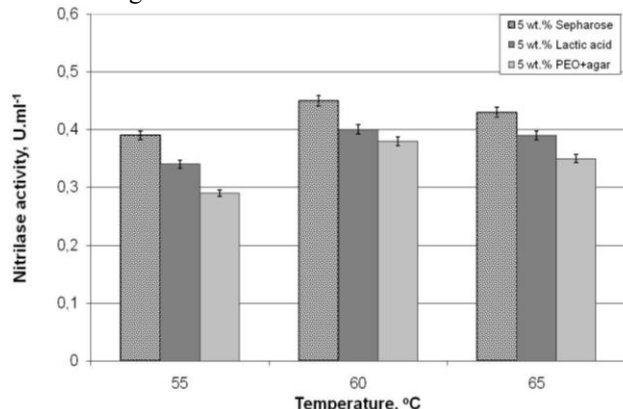


(b)

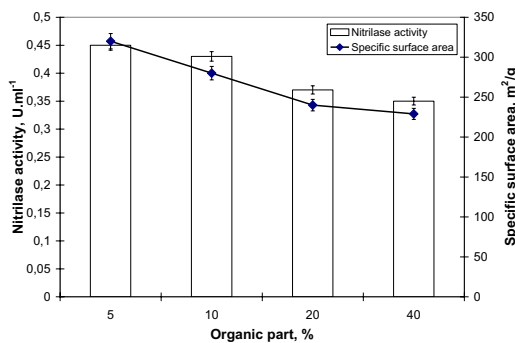


(c)

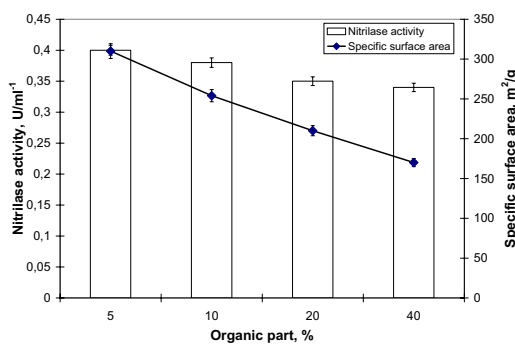
**Fig. 3.** Comparison between the activity obtained by the immobilized cells at multiple use with matrices with different organic constituent.



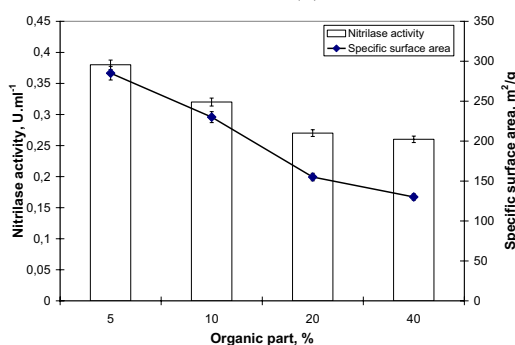
**Fig. 4.** Influence of temperature on the nitrilase activity of immobilized *Bacillus* sp. UG-5B strain. Error bars represent a deviation obtained from the triplicate measurements.



(a)



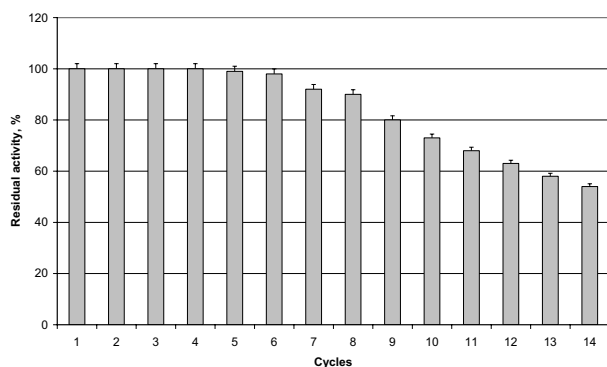
(b)



(c)

**Fig. 5.** Influence of specific surface area on the nitrilase activity of immobilized *Bacillus* sp. UG-5B strain (a-spharose; b-lactic acid; c-PEO+agar) Error bars represent a deviation obtained from the triplicate measurements.

Thus synthesized, the hybrid materials were tested as matrices for immobilization of the suspension of cells. Different parameters influenced the nitrilase activity of the immobilized biocatalyst. Their optimization was expected to lead to an enhanced enzyme activity compared to the free cells. The optimum pH for the nitrilase activity of free cells proved to be 6.0-8.0. The pH of the matrices was adjusted to 7.0-7.2 which appeared to be most appropriate for the cells entrapped in their volume. Figure 3 shows the comparison between the activity displayed by the immobilized cells at



**Fig. 6.** Reusability of the biocatalyst (TEOS and 5 wt.% sepharose) obtained after optimization of the immobilization parameters.

multiple use when each matrix contains only SiO<sub>2</sub> and different percentage of organic constituent. The addition of an organic part plays a favorable role when living objects are entrapped in the gel matrix. Concerning the type of the hybrid matrix, best results for *Bacillus sp.* UG-5B were achieved when immobilization was carried out in a matrix containing 5 wt. % sepharose. This fact can be explained by the different chemical composition and structure of the organic part included.

The obtained materials with 5 wt.% organic constituent with immobilized cells were tested for their behavior under different temperatures in the range from 50°C to 65°C (Fig. 4). Highest enzyme production for the immobilized preparations was obtained when the temperature maintained during the evaluated process was 60°C. The nitrilase activity only slightly decreases when temperature is increased to 65°C (0.43 U ml<sup>-1</sup> for the matrix with sepharose, 0.39 U ml<sup>-1</sup> when lactic acid is included in the hybrid matrix and 0.35 U ml<sup>-1</sup> when PEO+agar is present as an organic part), which is due to its thermostability [13]. Involving thermostable enzymes in industrial processes gives the advantage of a fast process and least contamination [14]. The nitrilase enzyme produced by *Rhodococcus rhodochrous* was found to be fairly thermostable at 45°C [8], while the nitrilase of *Bacillus sp.* UG-5B shows highest values at 60°C. Another parameter influencing the enzyme activity of immobilized preparations is the specific surface area of the hybrid materials (Fig. 5). The larger surface area promotes higher nitrilase activity when 5 wt.% of sepharose are present in the hybrid matrix (Fig. 5a). This is in correlation with the pore size, permitting free substrate penetration. Inclusion of 40 wt.% sepharose leads to lower nitrilase yield, corresponding to the lower specific surface area. The specific surface area decreases with the increase of the organic part

content, leading to a gradual increase in enzyme activity. Reusability was tested for the immobilized bacterial cells included in the most suitable matrix for highest nitrilase activity, namely, this containing 5% sepharose (Fig. 6). In this case the half-life of enzyme activity was retained for 14 reaction cycles, each carried out with the addition of a fresh toxic substrate (benzonitrile) for degradation. The proven reusability indicates an efficient process that is possible to be carried out at the optimal conditions for operation of the immobilized system.

## CONCLUSION

All hybrid materials used appeared to be appropriate carriers for the entrapped bacterial cells. The biocompatibility of the hybrid materials allows the bioactivity of cells to be retained. The obtained biocatalysts can be useful in enzymology and biotechnology and ecology, using the hydrolytic enzyme properties. Nitriles can cause harmful effects on human health, so there exists an urgent need for their removal. Immobilization of cells, producers of nitrilase can offer solution to the problems that appear with free cells, using their advantages - retention of catalytic activity, protection of cells against substrate inhibitory effects, as well as stability and increased enzyme yield, achieved by optimization of the factors, influencing the immobilization process.

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## ПАРАМЕТРИ НА ХИБРИДНИ МАТЕРИАЛИ, ВЛИЯЕЩИ ВЪРХУ ЕНЗИМНАТА АКТИВНОСТ НА ИМОБИЛИЗИРАНИ КЛЕТКИ

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(Резюме)

Хибридни материали, синтезирани по зол-гелен метод, бяха използвани като матрици за имобилизация на бактериални клетки, продуценти на ензима нитрилаза. В структурните изследвания на синтезираните хибриди бяха използвани различни методи: Инфрочервена спектроскопия (FT-IR), ВЕТ-анализ и атомно силова микроскопия (AFM). Проследено беше влиянието на структурата на тези материали върху техните свойства. Получените хибридни материали бяха успешно приложени за имобилизация на живи бактериални клетки, при което жизнеността им беше запазена и ензимните им системи запазиха своите функции в имобилизираните системи. Влиянието на различните параметри по време на процедурата на имобилизация беше оценено за оптимизация на процеса и увеличение на ензимната активност на изследвания ензим. Най-благоприятен ефект върху клетките на щам *Bacillus sp.* оказва матрицата с 5 wt.% сефароза и получената ензимна активност беше  $0.45 \text{ U ml}^{-1}$ , когато процесът се провежда при  $60^\circ\text{C}$ . Увеличената специфична повърхност на матрицата беше още един фактор, водещ до повишена нитрилазна активност.