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Sol-Gel Hybrid Materials Applied As Matrices For A Co-Immobilized System of Bacteria and Algae

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ABSTRACT

Sol-gel hybrids containing different quantity of algal polysaccharide were synthesized, characterized and used as matrices for immobilization of bacterial and algal cells. Cell-cell interaction in the prokaryote - eukaryote model of the unicellular microalga Scenedesmus acutus and bacterium Bacillus sp. UG-5B jointly immobilized in the hybrid material was evaluated. BET analyses revealed that introduction of polysaccharide and formation of a hybrid structure leads to a decrease in the surface area but to an increase in the pore size of the matrices. This matrix feature is inversely correlated with the enzyme activity of the bacterial strain. The use of hybrid sol-gel matrix for encapsulation of bacterial and algal cells was followed to prove its applicability for the synthesis of the enzyme nitrilase and the accomplishment of a nitrile degradation process. Sharp increase in the enzyme activity of the encapsulated bacteria was registered compared to this of the free cells when 5% of polysaccharide was included as an organic part. In the co-immobilization procedure performed, the symbiotic relationship of the bacterium and the algae are used to increase oxygen transfer rate to the bacteria by co-immobilizing it with algae for further increasing the enzyme production and 4-nitrobenzonitrile degradation.

Keywords: sol-gel method; algal polysaccharide; cell co-immobilization, 4-nitrobenzonitrile biodegradation

INTRODUCTION

The sol-gel silica and silica hybrid biomaterials have attached much attention due to their proved advantages: the mild experimental conditions of the synthetic method and the possibility to obtain many new materials with nanoscaled structure and extraordinary behaviours [1-9]. The silica hybrids synthesized by the sol-gel method are porous materials having pores large enough to allow diffusion. The pore size can

be easily controlled by changing the conditions during the sol–gel process. These properties are useful for immobilizing biomolecules while retaining their biological activities and allowing bioreactions to proceed inside the matrix [10-13].

Progress in cell immobilization over the past 40 years has resulted in a revolution in the use of biomolecules for selective extraction, delivery, separation, conversion and detection of reagents, biodegradation [14-16]. The use of biological species in these applications has typically relied on the successful immobilization of the intact biological reagent within or onto a suitable surface of hybrid nanomaterials [15].

Most nitriles are highly toxic and their microbial degradation is considered as an efficient way for detoxification of industrially polluted waters and soils [17]. The enzyme nitrilase (EC 3.2.1.21) achieves the direct hydrolysis of the toxic cyano-group containing substances, which are dangerous for human health and can be found in polluted waters and soils. The nitrile-metabolizing microorganisms are of profound interest for using their nitrilases as an alternative of nitrile hydrolysis to strong acids or base catalysts [18-20]. Therefore alternative physicochemical treatment technologies such as biological degradation, solvent extraction, adsorption have been recently introduced in literature [21]. The commercial viability of their nitrile converting enzymes for production of acrylamide and for the synthesis of nicotinamide, nicotinic acid from cyanopiridin or to carry out stereoselective transformations was also demonstrated. It is prudent to examine the benefits of mixed cultures under immobilized state to accelerate the bioprocesses [22]. There are several reports of co-immobilization of two or more cultures to derive the benefit of both cultures. Reilly and Scott [23] reviewed several cases of mixed-culture to derive various biochemicals. The co-immobilization of mixed cultures of algae and aerobic bacteria by encapsulation has solved the problems of oxygen limitation under high cell density conditions in the matrices [24, 25]. Wikstrom *et al*. [26] have co-immobilized *Chlorella vulgaris* with *Providencia* sp. in agarose, and employed them in the production of a -keto-isocaproic acid from l-leucine. It has been proved that the algae in the gel matrix acted as an *in situ* oxygen generator. Immobilized phototrophs can be also used to convert CO_2 into extracellular organic carbon that is usable by the bacteria, in that way increasing the biosynthetic functions of the bacteria and fungi [27].

The aim of the present study is to synthesize and study the structure of the sol-gel hybrids, containing different quantity of *Dixoniella grisea* polysaccharide (5 to 20 wt. %) and to follow its influence on the structure of the hybrid sol-gel matrix used for encapsulation of bacterial cells, degrading nitriles alone and in a co-immobilized form with a green algae.

MATERIALS AND METHODS

Matrix synthesis: The inorganic precursor tetramethylortosilicate (TMOS) was mixed with a buffered aqueous solution of the organic constituent- algal heteropolysaccharide in any desired ratio. In that way hybrid inorganic-organic composite materials were synthesized. The pH of the reaction was increased in order to increase hydrolysis rate by a phosphate buffer solution. No phase separation is observed before and after the gelation point. Gelation takes part within 30 minutes at room temperature. Thin transparent hybrid films were obtained.

The algal heteropolysaccharide (APS) used in this study was isolated from the red microalgae *Dixoniella grisea* (*Rhodophyta)* - strain UTEX LB 2320. Cell walls of algae possess polysaccharides [28]. The polysaccharide of *Dixoniella grisea* contains 9% sulphates. Its molecular weight is 5-7 KDa. The main monosaccharides in its composition are xylose, galactose and glucose. Glucuronic acid and semi-esterified sulphate groups can also be found which determines the acidic properties of the polymer. After intensive cultivation of the algal culture, the suspension undergoes centrifugation for 30 min at 6000g. Cells are removed. The supernatant is being precipitated with C_2H_5OH in a ratio 1:2. After this operation, the precipitated polysaccharide is collected by a needle. Dissolving in distilled water is carried out using a magnetic stirrer and after that dialysis was performed for 24h at 40°C.

The processes of sol-gel synthesis and structure evolution of gels have been studied by the following methods: XDR (X-ray PW1730/10 diffractometer, in the 2θ range, Cu-Kα radiation), BET (Gemini 2370 V5) and AFM (NanoScope Tapping Mode).

Strains: Cells of *Bacillus sp.* UG-5B (№ 8021) deposited in the National Bank for Industrial Microorganisms and Cell Cultures, Bulgaria), were harvested for 24 h on a rotary shaker in 100 ml flasks with nutrient medium, containing 20 mM 4-nitrobenzonitrile. The medium for cultivation contained (g/l): K_2HPO_4 2.0; NaCl 1.0; $MgSO_4$ 0.01; $FeSO_4$ x $7H_2O$ 0.02; biotin 2 9 10-5; thiamine, 0.004; inositol 0.002 and benzonitrile (20 mM), pH 7.2.
The green microalgae *Scenedesmus acutus* strain was cultivated in BBM (Bold`s Basal medium), which contained Mg l^{-1}: $NaNO_3$ -1250; $CaCl_2$.2H20 -2.5; KH_2PO_4 -175; K_2HPO_4 -75; $MgSO_4.7H_2O$ -75; ME -0.9; Soil extract -15000; EDTA -80.
Dixoniella grisea was acquired from the Austin University, Texas USA. It was isolated from brackish water in the region. It is maintained as a working culture in the laboratory collection of the Department of "Experimental Algology" of the Institute of Plant Physiology-Bulgarian Academy of Sciences. The culture medium used was of Brody and Emerson [29].
They were continuously illuminated (260 E m^{-2} s^{-1}) at temperature 26°C. Aeration was 100 $dm^3/m^3/h$ enriched with 2 % CO_2. The obtained algal culture at extensive cultivationwas was used for immobilization after centrifugation to reach a concentration of $5x10^6$cells ml^{-1}.
Photosynthetic capability was evaluated using the kinetics of PF, and recorded with the Multifunctional Plant Efficiency Analyzer M-PEA (built by Hansatech Instruments Ltd., King's Lynn, Norfolk, PE30 4NE, UK).

Immobilization procedures: Before performing the immobilization, the pH was adjusted according to the value needed to preserve the vitality of both strains used in the experiment: it was raised up to 7.0 for keeping cell vitality. The bacterial strain *Bacillus sp*. UG-5B, producing nitrilase were separated from the culture medium by centrifugation and then resuspended in phosphate buffer pH 7.2. Ssuspension of cells with a density of 35 mg.ml^{-1} cells was used in the immobilization process. 5 ml of this suspension was introduced into the sol-mixture. Culture suspention of the green microalgae *Scenedesmus acutus* strain Tomaselli 8 (Chlorophyta) was used for co-immobilization. For performing the co-immobilization 2.5 ml of the bacterial suspension and 2.5 ml of the algal suspension were introduced into the mixture before gelation.

Nitrile biodegradation: The nitrilase action was assayed by measuring the ammonia released according to the phenol hypochloride method of Fawcett and Scott (1960) [30]. One enzyme unit (U) is defined as the amount of enzyme, producing 1μmol ammonia min-1 at pH 7.2, 50°C and 20 mM substrate. Cell density was measured spectrophotometrically at 660 nm in relation to the dry weight according to the standard curve previously prepared.

RESULTS AND DISCUSSION

For the development of hybrid nanocomposites by the sol-gel method a mixing of inorganic precursors with polymer organic molecules should take place and during the setting between inorganic and organic components covalent chemical bonds, hydrogen bonds and electrostatic interactions can appear [31, 32].
The structure of the obtained hybrid materials was investigated involving different methods. The results of these studies give complete information about the evolution processes of the structure formation in the synthesized materials. The results from the XRD analysis showed that all the studied hybrids are amorphous and difference in the intensity and sharpening of the amorphous halo was observed. The characteristic peak at around 20 2-Theta corresponds to the siloxane network.

All materials have also been studied by means of BET analysis (fig. 1). The obtained results reveal that the introduction of polysaccharide leads to decreasing of the surface area, but to an increase in the pore size. The samples with 5% APS have a surface area around 450 m^2 g^{-1} and pore size around 1.3 nm. In the hybrids containing 20% APS the surface area decreased to 280 m^2 g^{-1} but the pore size is bigger (around 3 nm).

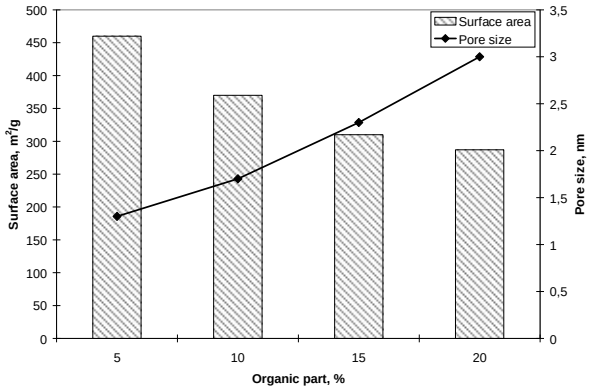
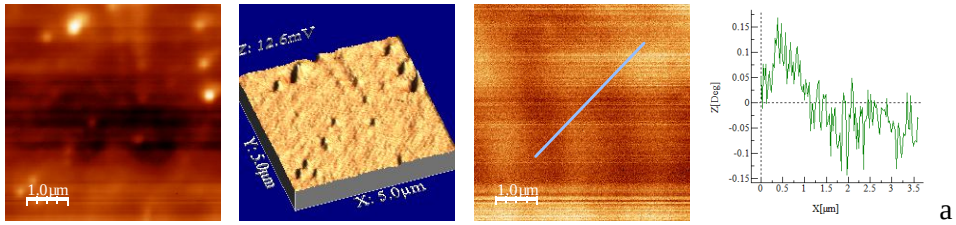


Figure 1. BET analysis reveals the correlation between pore size and average surface area of synthesized hybrid materials

The surface of obtained hybrids was structurally investigated with Atomic Force Microscopy (fig. 2). The presence of a hybrid nanostructure with well-defined nanounits and their aggregates, with different design formed by self-organizing processes was observed. The size of nanoparticles is up to 10 nm. The results gave the height distribution profiles of surfaces roughness. The histograms of the surface height distribution profiles, obtained from AFM images, showed that the inorganic-organic hybrid sample had a surface with irregularities of a small height. In the samples the nanoparticles are evenly distributed in the entire hybrid matrix and structures as clusters are visible.

Multiple atomic force microscopy images for each sample were obtained with the scan sizes: 3x3 μm. For the data interpretation the free-ware program WSxM was applied. Among other capabilities, the program allows the user to perform 3D rendering, pseudocolor image representation and roughness analysis. The representative AFM images of the hybrid surfaces, processed with the WSxM program, are represented in 3D. The plots for various scanned areas demonstrate a size of particle distribution based on the height measure (in the nanometer scale), and clearly show that height is different for the different samples. The minimum "height" is observed for hybrids with 5% APS. On the contrary, the largest height of pores is observed for the hybrids containing 20% APS. RMS roughness for each sample are: for the sample with 5% APS – 16.36 nm; for the sample with 10% APS – 19.54 nm; for the sample with 15% APS – 28.54 nm for sample with 20% APS – 35.21 nm, respectively.



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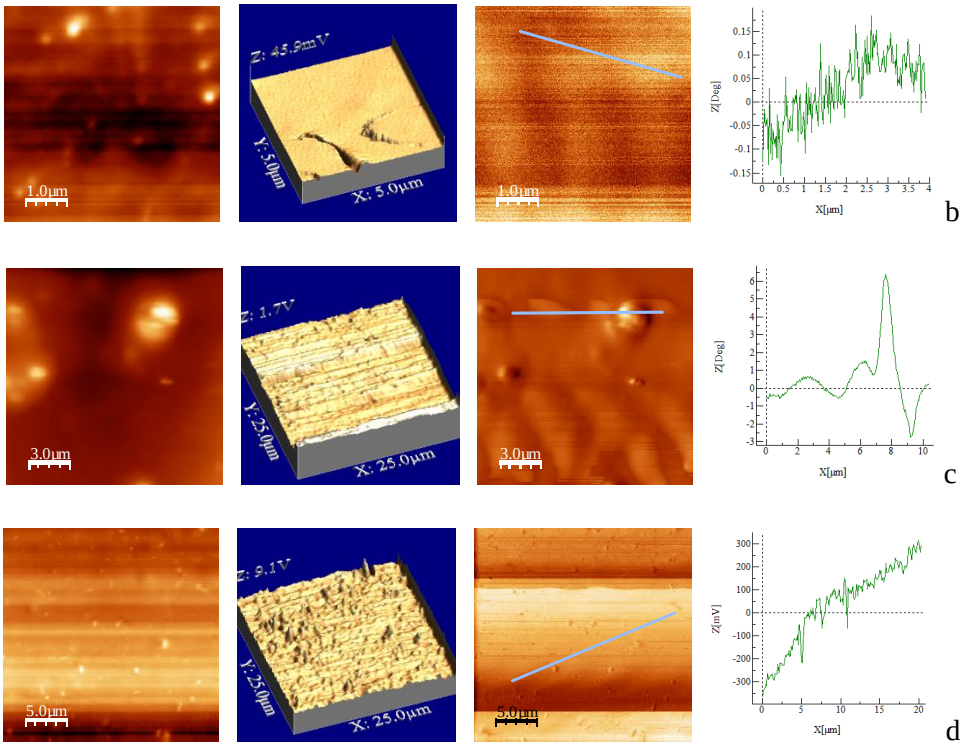

Figure 2. AFM image and height distribution profile of surface roughness of hybrid material containing TMOS and 5 % (a) 10 % (b), 15 %(c) and 20 % (d) algal polysaccharide

Cell immobilization has been extensively utilized for the degradation of toxic compounds in wastewater treatment processes and other biotechnological processes [33-35]. Immobilization methods include matrix entrapment and encapsulation.

Before accomplishing the co-immobilization procedure, two strains of algae were tested for their photosynthetic activity in an immobilized form. Better vitality and activity of the photosynthetic apparatus was established for *Scenedesmus acutus* compared to *Dixoniella grisea* (fig. 3). In the next steps the *Scenedesmus* strain was involved. Its activity was used as oxygen supplier to the bacterial cells.

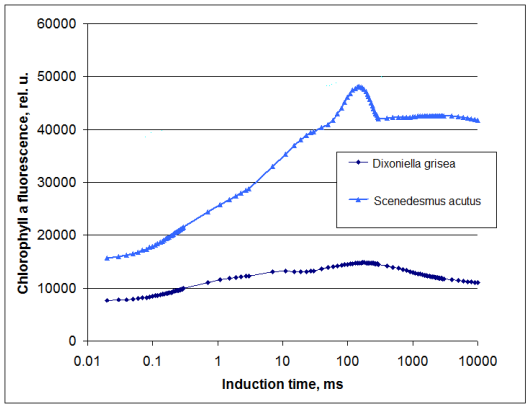

Figure 3. Prompt fluorescence of chlorophyll a for the two immobilized algal strains

The obtained hybrid materials were applied as carriers for entrapment of cells, producing the hydrolytic enzyme nitrilase. The nitrilase activity of the entrapped bacterial cells was evaluated. Sharp increase in the enzyme activity of the encapsulated bacterial cells alone and together with algae was registered compared to the activity of the free cells, especially when the percent of organic part is 5% (table 1).

Table 1. Relative nitrilase activity of free, immobilized and co-immobilized cells performing degradation of 4-nitrobenzonitrile

Substrate for degradation	Percent of organic part (APS) in the hybrid matrix with TMOS	Relative nitrilase activity, %		
		Free cells	Immobilized cells	Co-immobilized cells
4-nitrobenzonitrile	0%	12	14	18
4-nitrobenzonitrile	5%	12	36	71
4-nitrobenzonitrile	10%	12	29	64
4-nitrobenzonitrile	15%	12	27	61
4-nitrobenzonitrile	20%	12	25	56

As a control (100% enzyme activity) corresponds to the enzyme activity of the reaction carried out with 20 mM benzonitrile as a substrate at 50°C.

The addition of an algal heteropolysaccharide leads to increased enzyme productivity due to its influence on the structure of the matrix as well as it serves as a nutrient to the cells. A co-immobilized mixed suspension of algae, *Scenedesmus acutus*, and bacteria, *Bacillus sp*. UG-5B, were studied with the purpose to increase the nitrilase production. The oxygen produced by the algae in the co-immobilized preparation favours a more effective nitrlase production.

Batch reusability of immobilized and co-immobilized system was proved by measuring the residual nitrilase activity at 12 cycles of nitrile degradation. Each was performed in fresh substrate medium. The co-immobilized preparation was also used in the 12 batch cycles, It showed higher residual enzyme activity than the separately immobilized bacterial suspension without registration of a significant loss of activity (fig. 4).

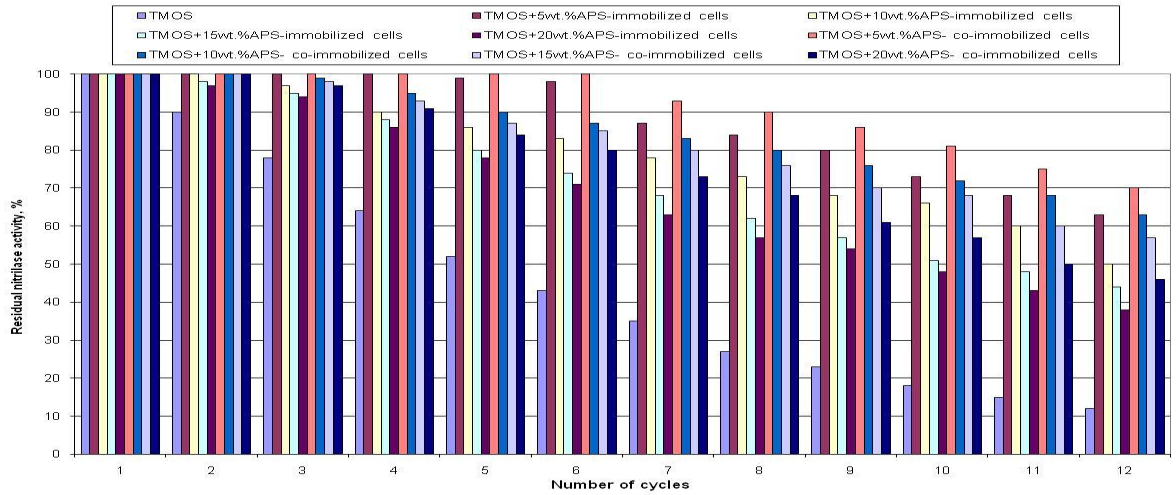

Figure 4. Residual nitrilase activity at re-use of immobilized and co-immobilized cells in hybrid matrices with different quantity of polysaccharide

To prove cell vitality and capability of reproduction, it was checked by disruption of the matrix with entrapped cells and their transfer into fresh nutrient medium. Cell growth was followed on the next and ensuing days spectrophotometrically and the results corresponding to the dry weight showed increase in the cell quantity. Cell concentration appeared to be 8 mg/ml on the first day, 15 mg ml^{-1} on the second and 19 mg L^{-1} on the third day.

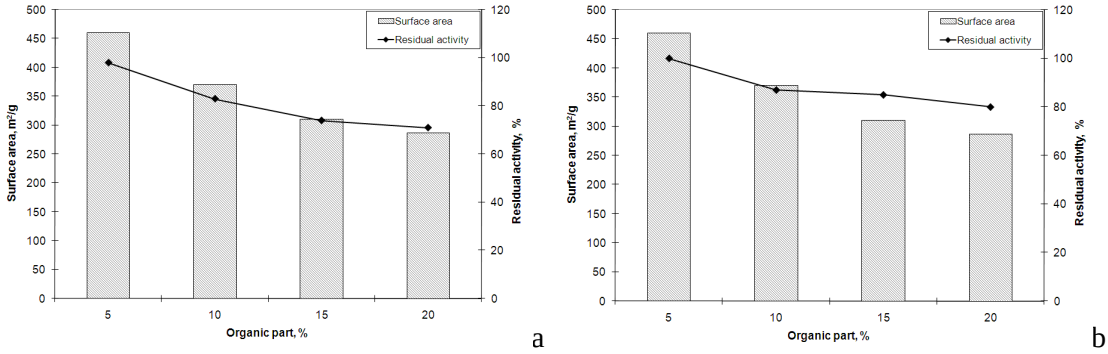

Fig 5. Relation between surface area of the matirces and residual nitrilase activity of the immobilized (a) and co-immobilized (b) system

A comparative survey was carried out to find the relation between the average surface area of the matrices and the residual nitrilase activity from the sixth cycle of operation (fig.5). The obtained results showed, that with the decrease of the average surface area the nitrilase activity also decreases.

APPLICATIONS

The synthesized materials by the sol-gel method were applied as matrices for co-immobilization of bacteria and algae in a system capable to perform nitrile compounds degradation.
Our experiments proved that cells captured in the sol-gel hybrid materials remain permanently entrapped and their relative nitrilase activity increased with 83,4%. Cell reproduction is not restricted and cellular organization is preserved upon encapsulation [36] Immobilization is carried out to avoid inhibition of high-concentration toxic chemicals on the cells, and provide a suitable microenvironment for biodegradation implementation. The microenvironment that is formed around the cells at encapsulation prevents to a certain extent the direct toxic action of the cyanide compound available in waste waters. The favourable effect of the addition of the algal polysaccharide in the hybrid sol-gel matrix was established in comparison to the pure silica matrix. Results from the experiments found in literature show that the viability of bacteria decreases quite quickly when they are trapped in a pure silica gel [37, 38]. The encapsulation and fixing biomolecules firmly without altering their activities is challenging for the utilization of biochemical functions of active biomolecules. Creating efficient biocatalysts includes immobilization processes leading to enhanced stability, improved specific activity, and increased product yield.

The proposed co-immobilization concept in which bacterial and algal cells are co-immobilized in a hybrid sol-gel matrix lead to an increased biodegradation of 4- nitrobenzonitrile catalysed by the bacterial nitrilase. It indicates the oxygen supply in the co-immobilized preparation was higher. A preparation consisting of only bacteria and no algae encapsulated was less effective. Repetition of the biodegradation process using the immobilized and co-immobilized systems revealed an effective biotechnological process with higher enzyme activity when the bacteria were immobilized together with the green algae, probably due to the increased oxygen content as well as the availability of some nutrients in the microenvironment. On the other hand, the favorable effect of the addition of the heteropolysaccharide in the sol-gel matrix used for immobilization helped to obtain high enzyme production. It influenced both the structure of the matrix and serves as a nutrient component for the cells.
Studies on the properties of the synthesized algal polysaccharides have rapidly developed during the last decades. Its antiviral and antitumor effects are well known [39-42]. In the present paper we showed another application of this valuable algal product- serving as an organic constituent in the hybrid sol-gel matrix, which proved to be an appropriate matrix for immobilization and co-immobilization of bacterial and algal strains.

CONCLUSIONS

The sol-gel hybrids, containing algal polysacharide were synthesized using the sol-gel method. The hybrid matrix was successfully applied for the encapsulation of the bacterial strain *Bacillus sp.* UG-5B, producer of nitrilase and the green microalgae *Scenedesmus acutus.* The comparison of the activity of entrapped in the hybrid matrix with algal heteropolysaccharide bacterial cells, proved the favorable effect of the polysaccharide. The biocompatibility of the hybrid materials allows cell structure to be preserved and bioactivity of both strains to be retained, enhancing the long-term stability and reusability of the obtained biocatalysts. They possess the potential to be involved in bioremediation processes for degradation of nitrile contaminants. The devlopment of a co-immobilized prokaryotic – eukaryotic organisms technology can provide new and effective waste water treatment methods.

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