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Research paper

Sapropel-based supports as novel macroporous carbon-mineral adsorbents for enzymatic active substances

G.A. Kovalenko [a,b,*], L.V. Perminova [a], N.A. Rudina [a], Yu.G Maksimova [c,d], A. Yu Maksimov [c,d]

[a] *Institute of Catalysis, 630090 Novosibirsk, Russia*
[b] *Novosibirsk State University, 630090 Novosibirsk, Russia*
[c] *Institute of Ecology and Genetics of Microorganisms, 614081 Perm, Russia*
[d] *Perm State University, 614990 Perm, Russia*

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Abstract

The novel macroporous carbon-mineral *Sapropel* supports were obtained from lacustrine sapropel silts of freshwater lakes by annealing of semi-coke in the inert atmosphere. The specific surface area of these supports varied from 10 to 100 m^2/g, the total pore volume from 0.3 cm^3/g till 1.6 cm^3/g; macropores of diameters more than 2 μm were predominating. The *Sapropel* supports were studied for the adsorption/adhesion of enzymatic active substances, such as whole bacterial cells, and invertase-active fully destroyed baker's yeast cells (autolysates), and purified enzyme nitrilase. The heterogeneous biocatalysts with required enzymatic activity were prepared and their properties were studied in the corresponding bioconversion processes. The invertase-active biocatalysts exhibited high activity, 120–135 U/g, and stability; the half-times of their inactivation ($t_{½}$) were more than 1000 h in the continuous process of sucrose hydrolysis at 50 °C. The nitrilase-active biocatalysts for "green" chemistry of nitriles possessed high activity, 350–500 U/g, and the $t_{½}$ were estimated to be more than 100 h in the periodic process of hydration of acrylonitrile to acrylic acid at 22 °C.

Keywords: Sapropels; Adsorption/adhesion; Biocatalysts; Nitrilase

1. Introduction

Environmental protection is a priority nowadays. R&D of the low-cost and highly efficient adsorbents using renewable raw materials or waste products as precursors is of great importance. Such adsorbents may be applied for the treatment of municipal waste and ground waters. For example, in order to remove As(III) and As(V) from aqueous solution, in particular from arsenic contaminated ground water, the adsorbents were derived from waste tire rubber as precursor. The dried and grinded rubber granules were pyrolyzed at 700°C in a tube furnace under the inert atmosphere of N_2 combined with steam [1]. The prepared adsorbents and its {alumina-on-carbon} composites were microporous; average pore radius were less than 2 nm, total pore volume was 0.04–0.07 cc/g, BET specific surface area was 100–180 m^2/g [1]. Biochar microparticles were prepared using waste materials, such as wheat straw, pine wood, pig manure and cardboard, and *Ephedra strobilacea* saw dust under pyrolysis at 450–500 °C in the presence of N_2 for 2–5 h [2–4]. These adsorbents were systematically studied in the process of adsorption and removal of methylene blue dye from aqueous solution under varying conditions [2–4].

Sapropels silt is renewable resources for production of cheap and available raw precursor materials and sapropels' recycling is a promising direction of the processing industry. There are huge deposits of sapropel silts on the bottom of freshwater lakes; nowadays the sapropel bed is estimated up to hundreds billion tons (up to 92 billion tons in Western Siberia). There are three main components in sapropels. Biologically active component is represented by amino acids, hormone-like compounds, enzymes, vitamins (carotins), sterols, chlorophyll, xanthophylls, as well as peptide-like materials [5,6]. These compounds were produced by microalgea (*Diatomeae*) and microorganisms inhabiting the lake silt. For instance, microbial communities of ancient Mediterranean sapropels are represented by a total of 98 pure cultures which could be grouped into 19 different phylotypes closely related to the

* Corresponding author. Institute of Catalysis, 630090 Novosibirsk, Russia. Fax: +7 383 330 80 56.
E-mail address: galina@catalysis.ru (G.A. Kovalenko).

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genera *Photobacterium* and *Agrobacterium* [7]. Organic matter of sapropel is the sum of bitumen, humic acids, cellulose, and lignin [8–10]. The composition of organic matter producing by dominating species of phyto- and zooplankton, and macrophytes, was determined, as well as sedimentation rate of plankton and phytodetritus was estimated [8]. Organic matter of sapropel contains 47–61% of carbon, 23–39 % of oxygen, 4–6 % of nitrogen and 5–9 % of hydrogen. Mineral component of sapropels is represented by SiO_2, Al_2O_3, CaO and Fe_2O_3, as well as oxides of Mg, K, S, P (1–5 wt%) and microelements such as Co, Mn, Cu, B, Zn, Mo, Cr, Ni, Ag, Sn, Pb, Hg. For example, sapropels of Black Sea contain ~50 wt% of SiO_2, 13–17 wt% of Al_2O_3, 5–8 wt% of CaO, ~ 6 wt% of Fe_2O_3, 2–3 wt% of MgO [11]. Anions in sapropels are represented by carbonates, phosphates, and sulfates.

The processing of lacustrine sapropel silts has attracted attention because of producing many valuable petrochemicals, in particular petroleum, crude oils, coke, coal gas, acetic acid, methanol, wax, and insulating materials. The crude oils generated from sapropels are characterized by low sulfur and less wax; the contents of benzene and dimethylcyclopentane are low; and hexane, cyclohexane and normal heptane have a high content [12,13]. Majority of research and engineering activities are concerned with the application of sapropel as a soil fertilizer in agriculture, as an inexpensive and disposable adsorbent in pollution control and environment protection, and as mud in balneology. Interest in processing of sapropel had a pause because of exploitation of rich oilfields, but now it is rekindled, notably for developing and obtaining novel effective and inexpensive supports or adsorbents [13].

Thermochemical processing of sapropels to produce semi-coke and coke is of great research and practice interest especially for treatment of petroleum pollution [13]. These solid products contained 9–64% of carbon and 36–91% of mineral component and possessed the following parameters: specific pore volume of 2–2.5 cm^3/g; average pore diameter of 0.2–2 μm, bulk weight density of 0.12–0.3 cm^3/g, granules' mechanical strength of 40–50 kg/cm^2. Petroleum capacity was equal to 1–18 g/g [13]. It is well known that low bulk weight density of petroleum absorbents (<0.1 g/cm^3) does not allow to provide the target application to polluted territory even under gentle breeze, but high bulk weight (>0.5–0.6 g/cm^3) results in the decrease of absorbent flotage during desired time (72 h), and therefore leads to the problems with its recovery off the water surface. As may be seen from parameters described above, the absorbents obtained on the basis of lacustrine sapropel with additives of peat and moss satisfied the requirements for usage in ecology. Undoubtedly, the solid products obtained by thermochemical processing of lacustrine sapropel (named *Sapropels*) are the basis to develop a novel type of supports for catalysts, various functional materials and composites for special purpose [13]. It should be noted that unlike the fine powders of biochars, in particular described in Refs. [2–4], *Sapropels* supports developed were macroporous supports with a high mechanical strength of granules of 0.5–3 mm in size. The macroporous structure allows *Sapropels* effectively bind various substances with a high molecular weight such as proteins and bacteria. Until now, the properties of sapropel have not been studied with respect to the adsorption/adhesion of enzymatic active substances in order to prepare highly stable heterogeneous biocatalysts with desired enzymatic activity.

In this work, *Sapropel* supports were first investigated as novel carbon-mineral adsorbents for enzymatic active substances, including whole cells of various bacteria (*Arthrobacter* sp., *Ralstonia* sp., *Rhodococcus* sp., recombinant *E. coli*,) and fully destroyed baker' yeast cells – invertase-active autolysates, and purified enzyme from *Pseudomonas fluorescens* – nitrilase. Adsorptive capacity of these supports, as well as enzyme activity and stability of the heterogeneous biocatalysts prepared by adhesion/adsorption of various enzymatic active substances were determined in the processes of glucose isomerization, and sucrose inversion, and acrylonitrile hydration.

2. Materials and methods

2.1. Sapropels

Adsorbents for this study were prepared from the starting material – lacustrine sapropel silts of Omsk freshwater lakes (Western Siberia) as described in Ref. [13]. At first, organic component of sapropel was destructed via carbonization at 300–350 °C. Then, semi-coke obtained was annealed in the inert atmosphere at 600–900 °C. This granulated supports were named as *Sapropels*. The chemical compositions of *Sapropels* and textural parameters were presented in Tables 1–4.

2.2. Enzymatic active substances

Active substances used in this study possessed various enzymatic activities and differed by nature and size. For instance, whole non-growing cells of bacteria (*Arthrobacter* sp., *E. coli*, *Ralstonia* sp.) were 1–2 μm in size. The diameter of enzyme molecule (nitrilase) did not exceed 10–20 nm. The enzymatic activity (A) of studied active substances was expressed in amount of units of activity (U); 1 U corresponded to the reaction rate of 1 μmol of substrate/product converted/generated per 1 min under the appropriate reaction conditions (temperature, pH) described below.

The specific activities of suspended whole bacterial cells and baker's yeast autolysates were expressed in number of U per 1 mg of dry substances. The concentration of dry substances was determined by measuring optical density (OD) at $\lambda = 590$ nm (1 cm-cuvette) and calculated using conversion coefficient from OD to concentration in mg of dry substances per 1 ml of suspension. The specific activity of soluble enzyme nitrilase was expressed in number of U per 1 mg of protein. The concentration of soluble protein was measured by Bradford' method [14] using bovine serum albumin as a standard.

Enzymatic active substances (EAS) were immobilized by adhesion/adsorption onto *Sapropels* and thereby heterogeneous biocatalysts with desired enzymatic activity were prepared. The activity (A) was expressed in amount of U per 1 g of the prepared biocatalyst. The value of A was calculated based on measuring desorption (leakage) of the EAS from support. Initially, the biocatalyst was placed in reaction media and the sum reaction rate (W_Σ) was measured under reaction conditions described below. Additionally, the rate of reaction was measured after removal of biocatalyst from reaction media ($W_{soluble}$).

In majority of experiments, $W_{soluble}$ did not equal to zero because the reaction continued due to EAS desorption, $W_{soluble} \geq 0$. The activity of the immobilized enzymatic active substances or the activity of biocatalysts (A) was calculated according to the formula $A = \frac{(W_{\Sigma} - W_{so;uble}) \cdot V}{m}$, where V – the volume of reaction media, m – the weight of dry support using for preparation of the biocatalysts, in gram. Experimental error did not exceed 10–15%.

2.2.1. Glucose isomerase active substances

As EAS having an activity of glucose isomerase (GI), two strain-producers of glucose isomerase were studied. Wild strain-producer *Arthrobacter nicotianae* described in Ref. [15] was used as a donor of gene *xylA* encoding glucose isomerase. Recombinant strain *E. coli* BL21 (DE3)*/pET24bxylA* (r*E. coli*) was used as a recipient of gene *xylA* and produced target glucose isomerase in amount ~50% of total intracellular proteins. Recombinant strain-producer of GI was studied in Ref. [16].

The glucose isomerase activity of suspended and adhered bacteria was determined under the following conditions: 70 °C, 1 мМ Mg^{2+}, 1 мМ Co^{2+}, 0.02 M phosphate buffer pH 7.8 and 7.0 for *A. nicotianae* and r*E. coli*, respectively. Substrates glucose and fructose were used in the concentration of 2 M. The GI activities of biomass of *A. nicotianae* and r*E. coli* in suspensions were approximately of 0.2–0.5 U and 3–4 U per 1 mg of dry cells, respectively.

2.2.2. Invertase active substance

As EAS having an activity of invertase (IN), autolysates of commercial baker's yeast were studied. The autolysis of yeast was carried out under stirring at 48 ± 2 °C for 18–22 h. Then autolysates were centrifuged at 5,000 rpm. After centrifugation supernatant was withdrawn and the sediment was suspended in 0.15 M KCl. This procedure was repeated 3–4 times until the supernatant becomes uncolored and transparent. The beige paste of yeast autolysates contained ca. 20 wt% of dry substances.

The invertase activity (IN) was determined at 50 °C in 0.05M acetate buffer pH 4.6. Substrate sucrose was used in the concentration 5–20 wt%. The IN activity of yeast autolysates in suspension was approximately of 15–30 U per 1 mg of dry substances.

2.2.3. Nitrilase active substance

As EAS having an activity of nitrilase (NI), this enzyme was isolated from the soil bacteria *Pseudomonas fluorescens* C2 (Perm region, Russia) and partially purified. Biomass of *P. fluorescens* C2 was grown and harvested at the stationary growth phase, then centrifuged at 10,500 × *g* for 20 min, washed and re-suspended with 44 mM sodium butyrate in 0.01 M phosphate buffer pH 7.2. Afterwards the suspension undergone sonication 7 times at 0–4°C for 20 s. Cell debris was removed by centrifugation (10,500 × *g*, 4°C, 20 min) and withdrawn. The supernatant containing the cell-free extract was exposed to 35% ammonium sulfate saturation, and then a precipitate was removed by centrifugation and re-suspended with 44 mM sodium butyrate in phosphate buffer. By this method, partially purified nitrilase was obtained and stored at −18°C.

The activity of nitrilase (NI) was assayed at 22°C in 0.01 M phosphate buffer pH 7.2. The concentrations of substrate–acrylonitrile varied from 0.07 M to 1.36 M. The reaction was stopped by adding HCl. The activity of soluble nitrilase was approximately of 5 U/mg of protein.

2.3. Immobilization of EAS on Sapropels

The adhesion of the non-growing bacteria onto *Sapropels* was carried out. For example, the adhesion of whole bacterial cells-producer of glucose isomerase such as wild strain *A. nicotianae* and recombinant strain r*E. coli* was performed by three methods. *Method 1* – adhesion of non-growing bacteria from cells' suspensions with concentration of dry substances of 1–120 mg/ml. The contacting of suspensions (10 vol. parts, 10 ml) with a support (1 wt. part) was carried out at ambient temperature for 1–2 days. *Method 2* – adhesion of non-growing bacteria from the highly concentrated cells' suspensions with concentration of dry substances of 1–2 g/ml, then drying of the adhered cells at 50 °C. *Method 3* – colonization of *Sapropel* by bacteria and adhesion of growing cells. By this method the bacteria was grown in the presence of *Sapropel* support and simultaneously adhered. All prepared biocatalysts were washed many times by buffer. Cross-linking was carried out as follows. *Sapropel M* (1 g) with adhered cells of *A. nicotianae* (1 g) was treated by 10 ml of 12.5% glutaraldehyde for 30 min at 18–20 °C. After that the biocatalyst were washed by buffer and dried.

The adhesion of baker's yeast autolysates was carried out under contacting of suspension in concentration 2–100 mg/ml with a support at a ratio of 10 (vol.part) to 1 (wt.part) at ambient temperature for 8 h.

The adsorption of the nitrilase was performed at 10°C for 24–145 h. The ratio of the volume of protein solution to the *Sapropel* weight was 5:1. Then the prepared biocatalysts were washed three times with 5 ml of phosphate buffer and stored at 10°C.

The adhesion value was determined as a difference of dry weight EAS before and after adhesion and expressed in mg of dry substances per 1 g of support. The amount of adsorbed protein was calculated from the difference of its concentration in a solution before and after adsorption and expressed in mg of protein per 1 g of the *Sapropel* support.

2.4. Determination of activity and stability of immobilized EAS

The packed-bed reactor was used for determining the activity and stability of the biocatalysts prepared by adhesion of the whole GI-active bacterial cells and IN-active yeast autolysates at 70 °C and 50 °C, respectively. The lab setup was composed: 1) temperature-controlled packed-bed reactor – glass column; 2) temperature-controlled chamber with reaction media mixed by magnetic stirrer; 4) thermostat; 5) peristaltic pump. Circulation rate of reaction media through biocatalyst bed was varied; commonly the flow rate was equal to 30 ml/min.

The stirred batch reactor was used for determining the activity and stability of the biocatalysts prepared by the adsorption

Table 1
Mineral components of *Sapropel* supports.

Supports	Carbon content, wt%	Mineral component, wt%			
		SiO_2	Al_2O_3	CaO	Fe_2O_3
Sapropel M	53.9	50.9	7.7	21.1	5.4
Sapropel L	63.9	55.1	7.6	9.1	6.7

of nitrilase on *Sapropel M*. The weight of the biocatalyst was 200 mg, the volume of reaction media was 5 ml. Concentration of acrylonitrile was 0.58 M. Duration of each reaction cycle was 24 h. After each reaction cycle the biocatalyst was washed by 5 ml of buffer and then used in the next cycle.

2.5. Analytical procedures

The texture parameters of *Sapropel* supports, in particular specific surface areas were measured by thermal desorption of argon using SORBI-Meta instrument (Russia), as well as adsorption/desorption of nitrogen using Quadrasorb evo (Quantachrome Instruments, USA). Pore size distributions were determined by mercury porosimetry using Micromeritics AUTO-PORE 9200 instrument. Scanning electron microscopic (SEM) studies were carried out using microscopes JEOL JSM 6460 LV and LEO 1430. The scale in the images corresponds to the distance in μm.

Monosaccharides (glucose and fructose) were analyzed by HPLC (Milichrom A-02, EcoNova, Russia) on inverted phase column ProntoSil 120-5C18AQ. At first, the 2,4-dinitrophenyl hydrazine derivatives of monosaccharides were obtained. Gradient elution in 0.1% CF_3COOH / (16–27%) CH_3CN was carried out at flow rate of 150 μl/min. The concentration of glucose was determined specifically by glucose oxidase method.

Concentration of acrylonitrile and acrylic acid was determined by HPLC (LC–10, Shimadzu, Japan) on a 250 mm × 4.6 mm Synergi 4u Hydro–RP 80A column, with UV detection at λ = 200 nm. The mobile phase was 25 mM NaH_2PO_4. The flow rate was 0.5 ml/min; the temperature of analysis was 25°C.

3. Results and discussion

3.1. Sapropels

The composition of sapropels depended on the freshwater lake where the deposit was located. Sapropels as raw materials for the studied *Sapropels* supports contained 36–83 wt% of organic matter. The element composition of this matter was following in wt % per dry substances: carbon – 42–54, nitrogen + oxygen – 20–33, hydrogen – 6–7, sulfur – 0.5–1.2. The mineral component of sapropels consisted of 51–67 wt% of SiO_2, 2–4 wt% CaO, 1–2 wt% Fe_2O_3, 3–6 wt% of SO_3, 0.1–0.7 wt% of P, 1.2–0.6 wt% of K. The compositions of mineral components and microelements of studied *Sapropels* supports were presented in Table 1 and Table 2.

The specific areas (S_{sp}) determined by adsorption/desorption of gases varied widely depending on gas (argon or nitrogen) and method of calculation (Table 3); an average value was estimated to be 20–40 m^2/g. It was found that the value of S_{sp} (N_2) increased two-fold when the size of granules decreased by an order, perhaps due to the presence of closed pores inside the granule. Adsorption equilibrium did not establish for 10 min of measurement point; N_2 adsorption and desorption curves did not coincide. Evidently, studded supports based on natural sapropels were very heterogeneous in structure.

The pore structure was determined by intrusion/extrusion of mercury (Table 4). The studied *Sapropels* were characterized by wide distribution of pore sizes. Large pores with diameter more than 1 μm by size were predominating. The volumes of macropores were estimated as 83% of V_Σ for *Sapropel A*, 76% of V_Σ for *Sapropel M* and 100% of V_Σ for *Sapropel L*. The

Table 2
Microelement composition of *Sapropel L*.

Element	Mn	Sr	Zn	Pb	Cu	Ni	Cr	Co	As	Hg	Mo	Cd
μg/g	1043.5	603.3	409.8	79.1	74.5	59.2	47.3	37.4	7.9	6.0	2.8	1.8

Table 3
Texture parameters determined by adsorption/desorption of gases (argon and nitrogen).

Support	$S_{sp\ BET}$ (Ar)*, m^2/g	$S_{sp\ MBET}$ (N_2)**, m^2/g	$S_{sp\ BJH}$ (N_2)**, m^2/g	$S_{sp\ DFT}$ (N_2)**, m^2/g	Pore diameter, D_{BJH}, μm
Sapropel M	33	45	12	113	3.2
Sapropel L	21	29	5	44	3.7

* The size of *Sapropels* granules was 1–2 mm.
** The size of *Sapropels* granules was 0.08–0.25 mm.

Table 4
Texture parameters of *Sapropels* determined by intrusion/extrusion of mercury.

Supports	Total pore area, m^2/g	Total intrusion volume, cm^3/g	Median pore diameter (volume), μm	Median pore diameter (area), μm	Average pore diameter, μm
Sapropel A	16	1.22	9.4	0.004	0.32
Sapropel M	13	0.51	12.8	0.007	0.16
Sapropel L	0.1	1.63	151.4	50.0	108.0

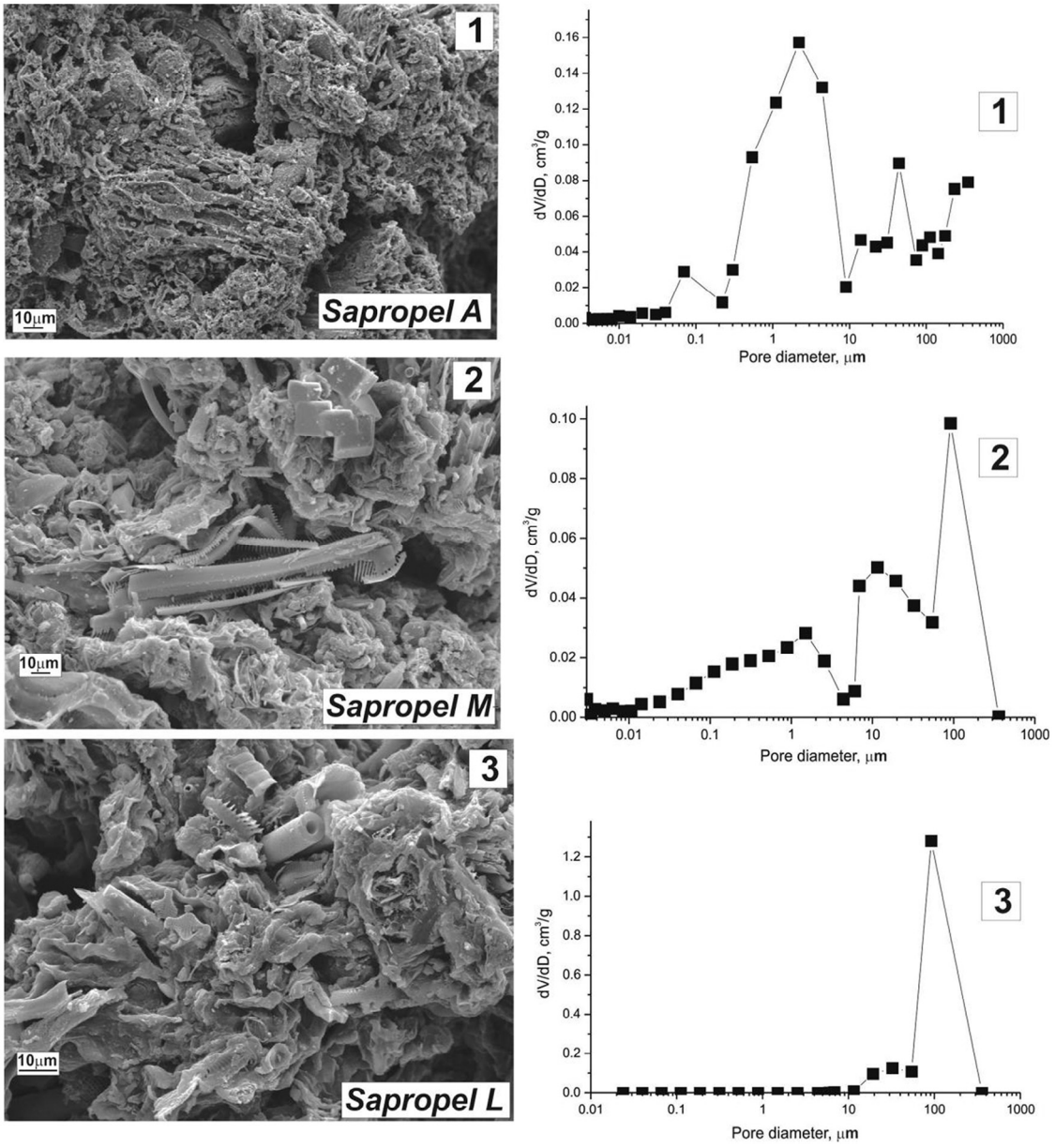

Fig. 1. SEM images of carbon-mineral supports and differential pore distribution curves for (1) *Sapropel A,* (2) *Sapropel M,* (3) *Sapropel L.*

volumes of mesopores of 10–100 nm were estimated as 5% for *Sapropel A* and 17% for *Sapropel M*. The volume of micropores did not exceed 1% of total pore volume of *Sapropel A* and *Sapropel M*. There were no mesopores and micropores in *Sapropel L*. As may be seen from Table 4, *Sapropels* were macroporous supports. The differential curve of pore distribution presented in Fig. 1 (on right side) confirmed this statement.

The morphology of the studied *Sapropel* supports was diverse and non-uniform as shown by the SEM images (Fig. 1, on left side). One can see the remains of microalgae and skeletons of plankton.

3.2. Adhesion of various bacteria and adsorption of enzyme onto Sapropels

It was found that the adhesion of bacterial cells on *Sapropels* depended strongly on taxonomy of microorganisms. For example, cells of *A. nicotianae* were adhered rather poorly on all solid supports including *Sapropels;* and slight adhesion *A. nicotianae* on various surfaces was due to the unique nature of these microorganisms not to the properties and adsorption ability of adsorbents [15]. Therefore, a peculiar method was developed for immobilization of such bacteria by immuring inside silica xerogel [17].

The amount of adhered *A. nicotianae* did not exceed 2 mg/g under conditions of *Method 1*. Taking into account the size of one bacterial cell (1–2 μm) and its weight (~10^{-12} g), calculations indicated that the measured amount of adhered bacteria (~1 mg/g) did correspond to the formation of dense monolayer of adhered cells on the external *geometric surface* of granules (0.5–1 mm in size) that was undoubtedly accessible for cells' adhesion. SEM image of adhered bacteria, in particular *Rhodococcus* and *Arthrobacter*, confirmed this calculation (Fig. 2, a, c). On the other hand, it was found that the bacteria

Table 5
Adhesion by *Method 1* of non-growing bacterial cells of GI strain-producers on *Sapropels* and initial glucose isomerase activity of the biocatalysts.

Support (granules 2–4 mm in size)	The adhesion, mg of dry cells/g of support		The initial GI activity, U/g of biocatalyst	
	A. nicotianae	r*E. coli*	*A. nicotianae*	r*E. coli*
Sapropel A	1.1	6.0	trace	4.8
Sapropel M	0.6	1.9	2.1	2.7
Sapropel L	0.3	5.4	trace	10.3

adhesion decreased from 1–2 mg/g to 0.3–0.6 mg/g (by a factor of 2–3) with increasing support granule size from 0.5–1 mm to 2–4 mm, though the geometric surface of granules (25 and 36 cm^2/g, correspondingly) increased by a factor 1.4. Thus, not only external geometric area but also internal surface of macropores inside granules was accessible for cell adhesion. The maximal adhesion of *A. nicotianae* (18 mg/g) was observed for *Sapropel A*, and the adhesion did not depend on granule size, i.e. adhesion occurred predominantly inside porous granules. Adhesion of recombinant *E. coli* cells was 3–18-fold greater than the adhesion of *A. nicotianae* (Table 5). According to the SEM image, there was no dense layer of non-growing r*E. coli* cells adhered by *Method 1* on geometric surface of *Sapropels L* granules (Fig. 2, b). Evidently, the bacterial cells diffused inside granules of this super-macroporous *Sapropel L*. One can see the adhered single whole cell (indicate by arrows) and perhaps the cell debris looks like as cobweb (Fig. 2).

As mentioned above, the adhesion of *A. nicotianae* on solid supports were weak and slight. The desorption (leakage) of bacteria was determined by measuring the reaction rate after removal of the biocatalyst from media, sometimes $W_\Sigma \approx W_{soluble}$.

It was found that bacterial cells of hydrogenase-active *Ralstonia eutropha* can adhere inside the macropores of *Sapropel M*. The dense layer of adhered cells was not observed on external surface of the adsorbent (Fig. 2, d). The adhesion reaches the value of ~3 mg of dry cells per 1 g of *Sapropel M* (Fig. 3 a).

It was found that *Rhodococcus* sp. adhered strongly onto *Sapropels*. As recently described in Ref. [18], the adhesion of non-growing nitrile hydratase-active cells of *R. ruber* gt1 on *Sapropel M* was close to 25 mg of dry cells/g. Taking into account the size of one dry bacterial cell *R. ruber* gt1 (1–2 × 7 μm) and its weight (~10^{-12} g), calculations indicated that all adhered cell occupied an area of 0.04 m^2/g. The surface area of pores more than 7 μm was determined by mercury porosimetry and was equal to 0.046 m^2/g. Thus, the dense layer of adhered cells of *R. ruber* was formed on the accessible area of *Sapropel* (Fig. 2a).

Macroporous *Sapropel A* was studied for adhesion of baker's yeast autolysates. The maximal adhesion value was 9 mg of dry

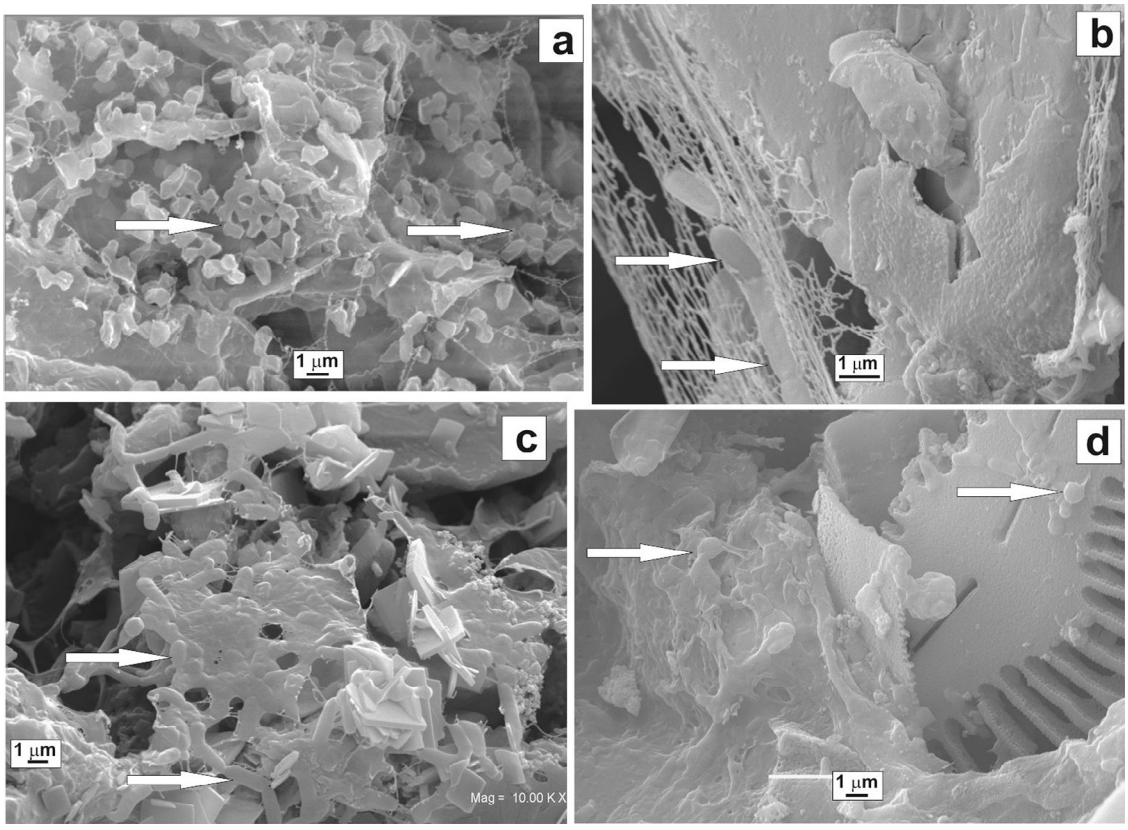

Fig. 2. SEM images of adhered cells of (a) *Rhodococcus ruber* on *Sapropel A*, (b) *rE.coli* on *Sapropel L*, (c) *Arthrobacter nicotianae* on *Sapropel M* after cultivation in the presence of adsorbent, (d) *Ralstonia eutropha* on *Sapropel M.* Arrows indicate the bacterial cells adhered.

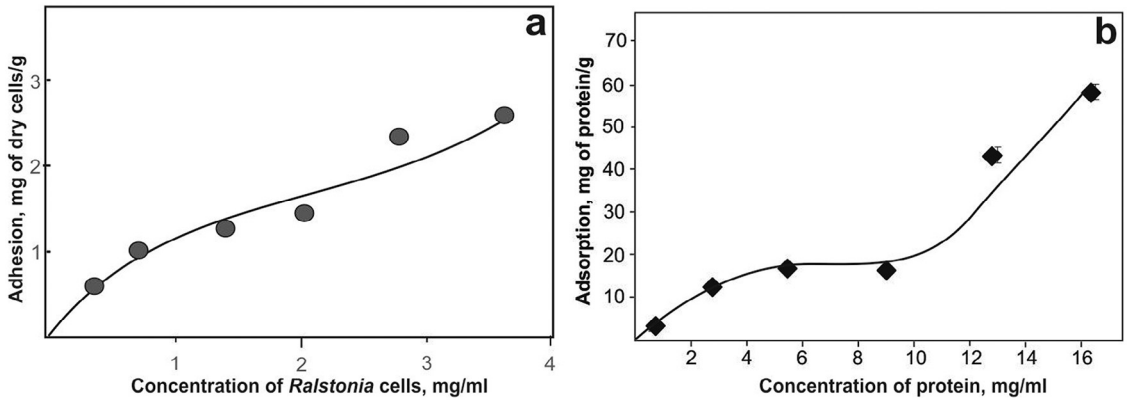

Fig. 3. Adsorption of (a) bacteria *Ralstonia eutropha* and (b) nitrilase from *Pseudomonas fluorescens* on *Sapropel M* in dependence on initial concentration of enzymatic active substances.

substances per 1 g of support. The adhesion was tight enough, $W_{soluble} \approx 0$.

Sapropel M was studied for adsorption of enzyme nitrilase due to the presence of mesopores which corresponded to the size of the enzyme molecule. Indeed, the volume of mesopores in *Sapropel M* was estimated to be approximately 18% of total pore volume. The kinetics of adsorption indicated that steady state in the system "enzyme solution/support" was established after more than 72 h. The duration of adsorption equal to 120 h was selected for adsorptive immobilization of this enzyme. The S-shaped isotherm indicated that the adsorption of enzyme at a concentration above 9 mg/ml was multilayered; the amount of adsorbed protein run up to ~60 mg/g of the support at the initial concentration of 16.5 mg/ml (Fig. 3b), i.e. 70% of protein molecules was adsorbed on the surface of *Sapropel M.* The adsorption value corresponding to monolayer was estimated as ~20 mg/g (Fig. 3b). It is known that microbial nitrilase consists of 6–26 identical subunits of 40 kDa by weight. Taking into account the value of the accessible area of *Sapropel M* and amount of adsorbed enzyme (in monolayer), the diameter of adsorbed one subunit was calculated to be more than 70 nm. This value is too large and exceeds ~4 times the common size of protein molecule with identical molecular weight. Probably, deformation of enzyme molecules occurred during immobilization on the carbon-mineral surface of *Sapropel M*.

3.3. Properties of the biocatalysts with glucose isomerase activity

It was found that the procedure of adhesion (*methods 1–3)* of whole cells *A. nicotianae* on *Sapropel M* had no influence on the biocatalyst properties. Thus, the initial GI activity was quite similar and close to 2 U/g, the half-times of inactivation ($t_{½}$) did not exceed 4 h at 70 °C. Biocatalysts prepared by adhesion of r*E. coli* onto *Sapropels* possessed the higher activities (Table 5) and stability; $t_{½}$ ~ 15 h at 70 °C for biocatalysts based on *Sapropels A* and *M* (Fig. 4a). According to the data in Table 1 and Fig. 1, *Sapropel L* had the largest size of pores (10 μm and more). The adhesion of bacteria on this support was the weakest, and the stability of biocatalyst was the smallest (Fig. 4a). As mentioned above, the main reason of relatively quick inactivation of GI-active biocatalysts was desorption

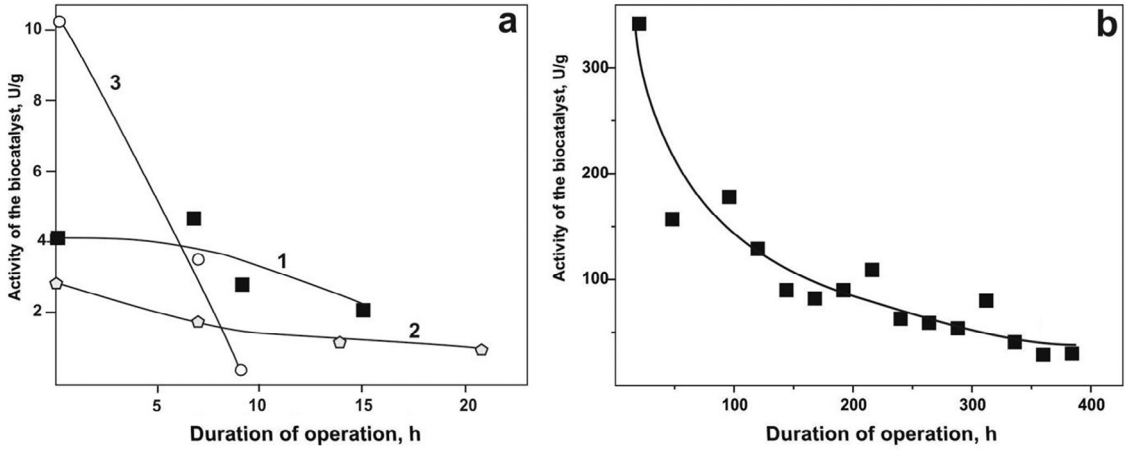

Fig. 4. Inactivation curves for (a) the biocatalysts prepared by adhesion r*E.coli* cells on various supports such as (1) *Sapropel A,* (2) *Sapropel M* and (3) *Sapropel L* in the reaction of fructose isomerization at 70 °C, and (b) the biocatalysts prepared by adsorption of nitrilase on *Sapropel M* in the periodic process of acrylonitrile hydration at 22 °C.

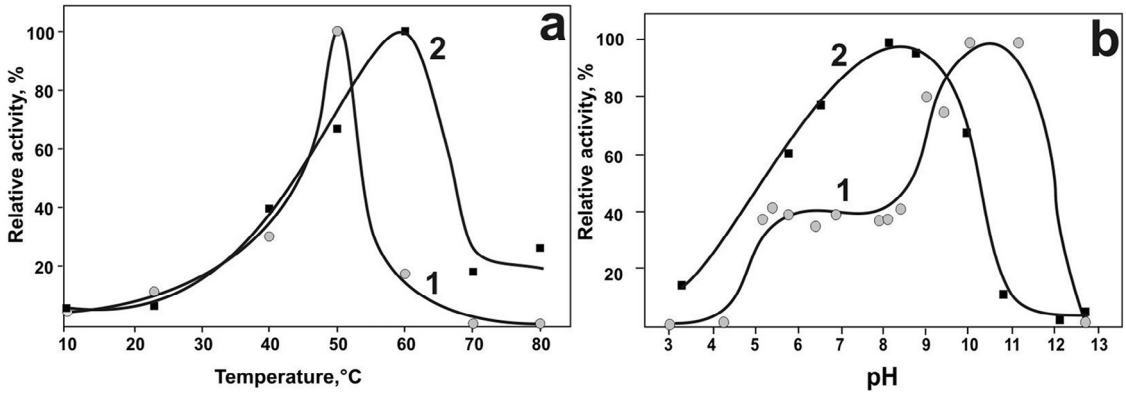

Fig. 5. The dependence of nitrilase activity on (a) temperature and (b) pH for (1) soluble nitrilase and (2) nitrilase adsorbed on *Sapropel M*

(leakage) of bacteria because of their weak adhesion. Indeed, the high reaction rate was measured in reaction media after biocatalyst removal, sometimes $W_{\Sigma} \approx W_{soluble}$. The decrease of leakage was observed under the cross-linking of *A. nicotianae* on *Sapropel M* by glutaric dialdehyde. But such cross-linking resulted in retaining only 4% of initial GI activity because of pronounced inactivation effect of this reagent.

3.4. Properties of biocatalysts with invertase activity

Macroporous *Sapropel A* was studied for adhesion of baker's yeast autolysates. The adhesion value was 6–9 mg/g. The invertase activity was completely retained after adhesion of yeast autolysate and even increased (by a factor of 1.3–1.4). For example, the specific activity of immobilized autolysates was found to be 19 U per 1 mg of dry substances, whereas activity of suspended autolysates was 15 U/mg. The highest invertase activity of 135 U per 1 g of biocatalyst was observed; the $t_{½}$ ~ 1440 h at 50 °C. The conversion of sucrose (5 % solution) was equal to 60% per 1 h at flow rate of 30 ml/min through packed bed of biocatalyst. The long-term stability was characterized by $t_{½} > 12$ months during storage in acetate buffer pH 4.6 at ambient temperature. The IN-active biocatalysts were defended by RU Patent [19].

As a conclusion, macroporous *Sapropel* supports were shown to be suitable support for adhesion of baker' yeast autolysates in order to prepare highly active and stable biocatalysts with invertase activity for production invert syrups.

3.5. Properties of biocatalyst with nitrilase activity

Nitrilase is very important enzyme for "green" chemistry of nitriles. *Sapropel M* with meso- and macropores was chosen for adsorption of this enzyme. It was found that immobilization of nitrilase resulted in 10-fold decrease in specific enzyme activity in comparison with one of soluble enzymes. This was probably caused by both deformation and/or unfavorable orientation of enzyme molecules on the carbon-mineral surface, but not diffusion limitations for mass transfer of substrate inside macropores. As mentioned above, the atypically large size of one adsorbed subunit calculated above may indicate a significant deformation of immobilized enzyme.

The kinetic parameters for soluble and immobilized nitrilase were determined by using Lineweaver-Burk plot and compared with each other. The Michaelis constants K_M for the acrylonitrile were found to be equal to 0.59 M and 0.23 M for soluble and immobilized enzyme, respectively. The decrease of apparent K_M after adsorption could be related to the effect of distribution of substrate in the microenvironment of immobilized enzyme.

The temperature-activity profiles were different for the soluble and immobilized nitrilase. The soluble nitrilase exhibited the highest activity at 50°C, whereas the optimum temperature for the adsorbed nitrilase was 60°C (Fig. 5a). Soluble nitrilase was found to be inactivated at 70°C, whereas immobilized nitrilase retained 18–20% of its activity. We may explain these results by adsorption of nitrilase inside mesopores corresponding by size to protein molecule; the increase of stability was owing to multipoint Van-der-Vaals interaction and enhancement of structure rigidity of the adsorbed enzyme molecules.

The pH-activity profiles were different for the soluble and adsorbed nitrilases as well. The optimum pH of soluble and immobilized nitrilase was 10.0–11.0 and 8.0, respectively (Fig. 5b). The pH-optimum shift may be caused by pH changing in the microenvironment of immobilized enzyme because of high content of mineral component (~46 wt%) in composition of *Sapropel M*. The soluble enzyme was inactivated at pH 3.0, whereas the adsorbed nitrilase retained about 14 % of the maximal activity. Both soluble and immobilized enzymes were inactivated at pH>12 (Fig. 5b).

The high initial nitrilase activity of the prepared biocatalysts exceeded 500 U/g at 22 °C. Stability of nitrilase absorbed on *Sapropel M* was determined for 16 batch reaction cycles (Fig. 4b). It was found that the loss of the activity was fitted by exponential decay obey with the inactivation constant equal to 0.011 h^{-1}; the half-life time was estimated to be 100 h (Fig. 4b).

4. Conclusions

Novel *Sapropels* supports were obtained by thermochemical processing of lakustine sapropels silts of freshwater lakes, in particular by annealing of semi-cokes in the inert atmosphere at

600 °C. These carbon-mineral supports were macroporous ones and were investigated as promising adsorbents for immobilizing enzymatic active substances, including whole bacterial cells, cell compartments (lysates), and enzymes in order to prepare the stable heterogeneous biocatalysts with required enzyme activity.

The biocatalysts for sucrose inversion prepared by adhesion of baker' yeast autolysate on *Sapropels* possessed high invertase activity (120–135 U/g) and high stability; half-life time ($t_{½}$) was more than 1000 h in continuous process of sucrose hydrolysis at 50 °C, and $t_{½}$ > 12 months under the storage at ambient temperature.

The absorbed nitrilase from *Pseudomonas fluorescens* had increased thermal stability and demonstrated the activity at more acidic pH as compared with the soluble enzyme. The initial activity of the nitrilase-active biocatalyst exceeded 500 U/g and multifold reuse of immobilized enzyme was carried out. The biocatalyst' half-life time was estimated at 100 h in the process of hydration of acrylonitrile to acrylic acid at ambient temperature.

As a conclusion, macroporous *Sapropels* supports seemed to be promising supports for adhesion of various microorganisms in order to prepare commercially attractive whole-cell biocatalysts. As well, the novel carbon-mineral *Sapropel* supports were found to be efficient adsorbent for the immobilization of enzymes, in particular nitrilase from *Pseudomonas fluorescens*. The nitrilase-active biocatalysts were promising for "green" chemistry of nitriles, for example, for acrylonitrile hydration to acrylic acid.

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References

[1] M.S. Karmacharya, V.K. Gupta, I. Tyagi, S. Agarwal, V.K. Jha, Removal of As(III) and As(V) using rubber tire derived activated carbon modified with alumina composite, J. Mol. Liquids 216 (2016) 836–844.

[2] G. Li, W. Zhu, C. Zhang, S. Zhang, L. Liu, L. Zhu, et al., Effect of a magnetic field on the adsorptive removal of methylene blue onto wheat straw biochar, Bioresour. Technol. 206 (2016) 16–22.

[3] L. Lonappan, T. Rouissi, R.K. Das, S.K. Brar, A.A. Ramirez, M. Verma, et al., Adsorption of methylene blue on biochar microparticles derived from different waste materials, Waste Management 49 (2016) 537–544.

[4] S. Agarwal, I. Tyagi, V.K. Gupta, N. Ghasemi, M. Shahivand, M. Ghasemi, Kinetics, equilibrium studies and thermodynamics of methylene blue adsorption on *Ephedra strobilacea* saw dust and modified using phosphoric acid and zinc chloride, J. Mol. Liquids 218 (2016) 208–218.

[5] M.J.L. Coolen, H. Cypionka, A.M. Sass, H. Sass, J. Overmann, Ongoing modification of Mediterranean Pleistocene sapropels, Science 296 (2002) 2407–2410.

[6] H. Knicker, P.G. Hatcher, Sequestration of organic nitrogen in the sapropel from Mangrove Lake, Bermuda, Org. Geochem. 32 (2001) 733–744.

[7] J. Suess, B. Engelen, H. Cypionka, H. Sass, Quantitative analysis of bacterial communities from Mediterranean sapropels based on cultivation-dependent methods, FEMS Microbiol. Ecol. 51 (2004) 109–121.

[8] N.I. Yermolaeva, E.Y. Zarubina, R.E. Romanov, G.A. Leonova, A.V. Puzanov, Hydrobiological conditions of sapropel formation in lakes in the south of Western Siberia, Water Resources 1 (2016) 129–140.

[9] Z.V. Semenova, D.F. Kushnarev, M.A. Litvintseva, A.V. Rokhin, Humic acids from the sapropel of Lake Ochaul, Solid Fuel Chemistry 41 (2007) 129–133.

[10] E. Dmitrieva, E. Efimova, K. Siundiukova, L. Perelomov, Surface properties of humic acids from peat and sapropel of increasing transformation, Environmental Chemistry Letters 13 (2015) 197–202.

[11] G.N. Baturin, Geochemistry of sapropel in the Black Sea, Geochemistry International 49 (2011) 531–535.

[12] W. Xiang, Z. Min, L. Yuhua, Crude oil compositions and geochemical characteristics of light hydrocarbons in northern slope of Tazhong, Tianranqi Diqiu Kexue 19 (2008) 713–717.

[13] G.V. Plaksin, O.I. Krivonos, Thermochemical processing of lake sapropels: composition and properties of products, Ross. Khim. Zhurn. 51 (2007) 140–147.

[14] M.M. Bradford, A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye-binding, Anal. Biochem. 72 (1976) 248–254.

[15] G.A. Kovalenko, L.V. Perminova, T.G. Terent'eva, L.I. Sapunova, A.G. Lobanok, T.V. Chuenko, et al., Glucose isomerase activity in suspensions of *Arthrobacter nicotianae* cells and adsorption immobilization of the microorganisms on inorganic carriers, Appl. Biochem. Microbiol. 44 (2008) 174–181.

[16] G.A. Kovalenko, L.V. Perminova, T.V. Chuenko, L.I. Sapunova, E.A. Shlyakhotko, A.G. Lobanok, Immobilization of a recombinant strain producing glucose isomerase on SiO_2-xerogel and properties of prepared biocatalysts, Appl. Biochem. Microbiol. 47 (2011) 151–157.

[17] G.A. Kovalenko, L.V. Perminova, L.I. Sapunova, A peculiar method for immobilization of non-growing microbial cells by entrapment into silica xerogel, in: S.D. Varfolomeev, G.E. Zaikov, L.P. Krylova (Eds.), Biotechnology in Medicine, Foodstuffs, Biocatalysis, Environment and Biogeotechnology, NOVA Science Publishers, Inc., New York, 2010, pp. 41–49.

[18] Yu.G. Maximova, G.A. Kovalenko, A.Yu. Maximov, V.A. Demakov, T.V. Chuenko, N.A. Rudina, Immobilized non-growing cells of *Rhodococcus ruber* as heterogeneous biocatalysts for the process of hydration of acrylonintile to acrylamide, Catalysis in Industry 1 (2008) 44–50.

[19] G.A. Kovalenko, L.V. Perminova, G.V. Plaksin, Biocatalyst for sucrose inversion, support of biocatalyst, method of preparing biocatalyst and processing inversion of sucrose, RU Patent 2 279 475 C2. Publ. 10.07.2006.