Journal of Molecular Catalysis B: Enzymatic 76 (2012) 116–124

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Review

Immobilization of enzymatic active substances by immuring inside nanocarbon-in-silica composites

Galina A. Kovalenko*, Larisa V. Perminova, Nina A. Rudina, Ilya N. Mazov, Sergey I. Moseenkov, Vladimir L. Kuznetsov

Institute of Catalysis, Novosibirsk 630090, Russia

ARTICLE INFO

Article history:
Received 16 June 2011
Received in revised form 6 September 2011
Accepted 21 November 2011
Available online 15 December 2011

Keywords:
Immuring enzymatic active substances
Nanocarbon-in-silica composites
Heterogeneous biocatalysts

ABSTRACT

A comparative study of multi-component heterogeneous biocatalysts prepared by immuring enzymatic active substances inside the {nanocarbons-in-silica} composites was carried out. Carbonic materials such as nanotubes, nanofibers, and onion-like nanocarbon were examined for inclusion inside SiO_2-based biocatalysts. The properties of the biocatalysts prepared, such as enzyme activity and stability, were studied depending on the content, physicochemical properties and nanostructure of the nanocarbons included. The biomasses of recombinant strain-producer of glucose isomerase *Escherichia coli* (rec-*E.coli*) and of baker's yeast autolysates were used for biocatalysts' preparing. The direct correlation between magnitude of increase of biocatalysts' steady-state activity and efficiency of adsorption/adhesion of enzymatic active substances (enzyme/cell compartments) on nanocarbons was observed. In the case of weak adsorption of glucose isomerase on carbonic materials, the steady-state activity increased by a factor of 1.5 for the catalysts prepared by immuring rec-*E.coli* inside the {nanorarbons-in-silica) composites and "dry" cross-linking by glutaric dialdehyde (<1 wt%). In the case of tight adhesion of yeast autolysates on multi-walled carbon nanotubes, the steady-state invertase activity increased by a factor of 6 for the biocatalysts prepared by immuring autolysates inside {MWCNTs-in-silica} composites. The activity of these biocatalysts exceeded ~3000 U/g, and the half-life time was more than 250 h in the continuous sucrose inversion at 50 °C.

Contents

* Corresponding author. Tel.: +7 383 3269743; fax: +7 383 3308056.
E-mail address: galina@catalysis.ru (G.A. Kovalenko).

1381-1177/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2011.11.015

1. Introduction

Nowadays research and development on new types of composite materials containing nanostructured carbon (nanotubes, nanofibers, and nanospheres) and their practical applications are progressing rapidly. Having unique electrical and mechanical properties, carbon nanotubes are promising materials for production of electronic devices, electronics and composites seem to be the asked-for area for wide applications of these nanocarbons. Chemistry and catalysis are rather unexplored areas for nanocarbons usage up to date.

In the literature [1–11] we find few examples of the practical applications of nanostructured carbons in analytical chemistry. Nanotubes (CNTs) and its composites have received much consideration for their use in chemical sensors and biosensors [1]. Previously CNTs were used in the field-effect transistors for the analysis of gaseous compounds due to extremely high sensitivity of electrical properties of the CNT-semiconductor to analytes' adsorption. Prior to using in such sensors, the carbon nanotubes are modified in two-stage procedure: in the first stage, nanotubes are covered with polymer films; in the second stage, the polymer is functionalized by introducing various chemical groups. The direct modification of nanotubes themselves is rarely performed, since in that case the conducting properties of CNTs disappear. The interaction of analyzing compounds with a sensitive layer of polymers (molten organic salts and polyethylene oxide) located on the surface of nanotubes results in a change in the electrical properties of CNTs reliably detected [2,3].

Carbon nanotubes are widely used in amperometric biosensors. In this case, CNTs are included into the polymers and such composites are coating an electrode; permitting the overpotential to be reduced during the detection of many electrochemical active analytes. For example, a highly sensitive and specific oxalate biosensor was constructed by covalently immobilizing oxalate oxidase on {carboxylated CNTs-in-polyaniline} composite film electrodeposited over the surface of Pt-wire using carbodiimide's chemistry [4]. The optimized oxalate biosensor showed a rapid response within 5 s. Not only synthetic organic polymers but natural ones also are the objects of active study. For instance, the composite of gelatin hydrogel and multi-walled carbon nanotubes (MWCNTs) was considered in [5]. The bioelectrochemistry of hemoglobin immobilized inside {MWCNTs-in-alginate gel} composite film was described in [6]. Hemoglobin retained the native secondary structure, achieved direct electron transfer and revealed excellent bioelectrocatalytic activity to the reduction of hydrogen peroxide, and, finally, the biosensor for analysis of H_2O_2 concentration in real samples was designed. The effectiveness of the inclusion of functionalized carbon nanotubes into chitosan using different cross-linking reagents was evaluated in [7]. Another example is that the electrodes that comprised {CNT|Au-nanoparticles|Teflon} composite were used in biosensors for glucose; and the detection limit decreased significantly (by a factor of 2); and, more importantly, the sensor's serviceability increased up to 3 months [9]. In comparison with commercial Glucometers, the sensitivity of glucose analysis performed by CNT-based biosensors is considerably higher; the detection limit is 0.2–33 μmol, the linear range is 7×10^{-4}–3.5 mM (0.5–33 mM for Glucometers) [1].

In 2003, information was published regarding completely new types of composites, that is, {ionic liquid|carbon nanotubes} [10]. The mixing of an ionic liquid based on dialkylimidazole with CNTs caused the formation of a mechanically and thermally stable gel, even if the content of carbon nanotubes was only several wt%. The investigation of electrochemical properties of these kinds of gels revealed their high conductivity, which was even greater than the conductivity of graphite pastes conventionally used furthermore, a synergistic enhancement of conductivity was observed for these composites. The presence of mixed conductivity (electronic conductivity in nanotubes and ionic conductivity in liquids), a wide range of operating potentials in electrochemical devices and the possibility of immobilizing additional components, e.g. catalysts, enzymes, specific reagents, etc., offer considerable promise for the practical application of these composite materials in various fields, including analytics.

It should be noted that adsorptive properties of nanostructured carbons with respect to enzymatic active substances have thus far been inadequately studied in terms of quantity. The reason has to do with the great difficulties to explore these fine-powder materials in aqueous solutions. In [11], study on interaction of enzymes (glucose oxidase) and metal-containing proteins (cytochrome c) with single-walled carbon nanotubes (SWCNTs) was performed; the results demonstrated that the physical adsorption of proteins was tight and binding did not require covalent immobilization. When glucose oxidase was immobilized on CNTs, the sensitivity of the designed amperometric biosensor was increased by an order of magnitude [11].

In [12] we studied the adsorption/adhesion ability of the supports based on catalytic filamentous carbon (bulk CFC) with respect to amino acids (lysine), proteins (albumin), and bacteria (*Rhodococcus*). The granules of bulk CFC were formed via interlacing and compacting carbon nanofibers (CNFs) synthesized on supported Ni, Cu-catalysts by H_2–C_3–C_4 pyrolysis. It was found that the adsorptive ability of bulk CFC barely depended on carbon nanofibers' nanostructure ("fishbone" or "deck of cards"), but was generally defined by the accessible surface area and surface roughness of CFC-based adsorbents [12]. Undoubtedly, the nanocarbons and their composites seem to be of great interest and perspective, and we undertook the pioneer study on application these materials in heterogeneous biocatalysis.

This work is devoted to comparative study of multi-component heterogeneous biocatalysts prepared by immuring enzymatic active substances (EAS) inside {nanocarbon-in-SiO_2–xerogel} composites. The properties (enzymatic activity and stability) of the biocatalysts were studied depending on the content, physicochemical properties and nanostructure of the nanocarbons included, as well as on the origin of enzymatic active substances. In the case of the latter, the biomasses of recombinant strain-producer of glucose isomerase *E. coli*, as well as of baker's yeast autolysates were used.

2. Experimental

2.1. Materials

2.1.1. Enzymatic active substances

The recombinant strain-producer of glucose isomerase *E. coli* BL21(DE3)/*pET24bxylA*, rec-*E.coli*, was donated by researchers of Institute of Microbiology (Belarus, Minsk). The conditions of the growth of bacteria were described in [13]. For the preparation of biocatalysts, we used the microbial biomass collected at the end of the late logarithmic phase or at the beginning of the steady-state phase by centrifugation at 5000 rpm for 15 min. The glucose isomerase activity in cell suspension varied from ~2000 to 4000 U per 1 g of dry cells at 70 °C.

Yeast autolysates were obtained by commercial baker's yeast autolysis performed with continuous mixing for 18–22 h at 48 ± 1 °C, as described in [14]. The biomass was collected by centrifugation 5000 rpm for 30 min, a supernatant was withdrawn, and the sediment was then suspended in 0.02 M acetate buffer pH 4.6. This procedure was repeated for 3–4 times until the supernatant became transparent and colorless. A paste-like sediment of yeast autolysates was fawn-colored and contained 20–22 wt% dry substances; the invertase activity in suspension varied from ~26,000 to 32,000 U per 1 g of dry autolysates at 50 °C.

Table 1
Texture characteristic of nanocarbons and the biocatalysts prepared by immuring yeast autolysates inside {nanocarbon-in-silica} composites, as well as invertase steady-state activity of the biocatalysts.

Nanocarbon materials			Biocatalysts based on yeast autolysates		
Abbreviation	S_{sp} BET, m^2/g	Diameter of primary carbon nanoparticle, nm	Composite[a] {nanocarbon-in-silica}	S_{sp} BET, m^2/g	A_{st}, U/g
DWCNTs (a)	95	5–50	DWCNTs-in-SiO_2	–	780
MWCNTs (b)	320	9–11	MWCNTs-in-SiO_2	88	3051
MWoCNTs (c)	330	9–11	MWoCNTs-in-SiO_2	71	1370
OLNC (d)	485	5–6 (150 in aggregates)	OLNC-in-SiO_2	94	963
ND (e)	325	4–6 (200 in aggregates)	ND-in-SiO_2	81	794
CNFs (f)	162	20–60	CNFs-in-SiO_2	57	892
			Without carbon	91	646

[a] Content of nanocarbon is 15 wt%.

In order to calculate the concentration of suspended cells/autolysates (g/l), we measured the optical density (OD) of the suspensions at a wavelength of 590 nm and determined a equalizing coefficient, the value of which was 3.2 g/l/unit OD (l = 1 mm) and 1.3 g/l/unit OD (l = 1 mm) for rec-*E.coli* and autolysates, respectively.

2.1.2. Nanocarbon materials

As nanocarbons in the composite biocatalysts, the following carbonic materials were used: (a) double-walled carbon nanotubes (DWCNTs) synthesized in a quantity of 9 wt% by methane pyrolysis on Co,Mo/MgO catalysts [15]; (b) multiwalled carbon nanotubes (MWCNTs) synthesized by CVD ethylene decomposition over supported Fe,Co-catalyst [16]; (c) MWCNTs oxidized by reflux in nitric acid (MWoCNTs) as described in [17]; (d) onion-like nanocarbon (OLNC) synthesized by high temperature annealing in vacuum of nanodiamonds [18]; (e) nanodiamonds (ND, produced in Biisk, Russia) containing 85–91% carbon, and the primary particles (4–6 nm in size) are aggregated in 50–200 nm particles; (f) carbon nanofibers (CNFs) having a "fishbone" structure and synthesized by methane pyrolysis on supported Ni,Cu/Al_2O_3 catalysts [19]. Carbon materials (a–e) were fine powders. Granules of bulk CFC (f) were grinded to fine powder in a mortar prior to the preparation of biocatalysts. Physicochemical parameters, in particular texture characteristic of nanocarbons are listed in Table 1.

2.1.3. Silica hydrogel

Silica hydrogel was obtained via coagel formation in continuous sol–gel procedure using sodium silicate (liquid glass) reaction with ammonium nitrate at pH 7.5 and 70 °C as described in [20]. The hydrogel humidity was 80–90%. After drying silica hydrogel at 105–120 °C, silica xerogel (SiO_2–xerogel) possessed specific surface area equal to 260 m^2/g.

2.2. Methods

2.2.1. Preparation of the heterogeneous biocatalysts

The preparation of heterogeneous biocatalysts was performed via the immuring enzymatic active substances inside {nanocarbon-in-silica} composites, in accordance with the procedure described in [13,21]. For this purpose, the enzymatic active substances were thoroughly mixed with silica hydrogel and nanocarbons. For the biocatalysts based on rec-*E.coli*, insoluble cobalt hydroxides (Co_xO_y) were additionally included into composites with the purpose of increasing the stability of glucose isomerase. Then paste-like multi-component homogeneous mixture was dried to an air-dry state (the humidity was ca. 10%), mechanically grinded, compressed at pressure of 150 bar and finally fractionated in order to obtain granules of a dry solid biocatalyst from 0.2 to 4 mm in size.

For the biocatalysts prepared with rec-*E.coli*, "wet" cross-linking was carried out during mixing all components before drying biocatalysts as described in [13,21]. "Dry" cross-linking was performed during contacting of dry biocatalysts' granules (0.1 g) with a solution of 0.1–1 wt% glutaric dialdehyde (0.5 ml) at ambient temperature for 4 h.

2.2.2. Determination of the adsorptive ability of nanocarbons

The fine power of nanocarbons was compressed into pellet at pressure of 150 bar and fractionated in order to obtain granules of 0.1–0.2 mm in size. Adsorption of bovine serum albumin (BSA, Serva), glucose isomerase and adhesion of yeast autolysates was performed for 1 day at ambient temperature undergoing gentle periodic agitation of the granules in a solution/suspension with initial concentrations ca. 1–1.5 mg/ml.

2.2.3. Measurements of the activity and the stability of the biocatalysts

The measurements of the glucose isomerase (GI) activity were performed in a medium of 0.02 M phosphate buffer pH 7.0 at 70 °C. A 2–3 M fructose solution was used as a substrate. In order to determine the enzymatic activity in a cell suspension, ions of Mg^{2+} and Co^{2+} as sulfates with a concentration of 1 mM were added into the reaction medium. No Co^{2+} ions were added into the reaction medium for the measuring activity of the heterogeneous Co_xO_y-containing biocatalysts. The measurements of the invertase (IN) activity were taken in a 0.02 M acetate buffer pH 4.6 at 50 °C. As a substrate, a 0.56 M solution of sucrose was used. The rate of the reaction: 1 μmol/min, was taken to be one unit of the enzymatic activity (U). The activity (A) was expressed in U per 1 g of dry biomass, or in U per 1 mg of protein, or in U per 1 g of dry biocatalyst. The concentration of glucose produced during fructose isomerization or sucrose inversion was determined spectrophotometrically by glucose oxidase-method. The experimental error did not exceed by 15%.

The activity of the heterogeneous biocatalysts was measured using a circulation set-up consisting of (1) a differential gradientless reactor in the form of a glass column with a thin bed of the prepared biocatalyst (0.1–0.5 g); (2) a mixer on a magnetic stirrer; (3) a thermostat maintaining a given temperature of the reaction medium in the mixer and in the biocatalyst bed (50 °C or 70 °C); and (4) peristaltic pump providing the circulation of substrate solution through the biocatalyst bed with a flow rate 1–35 ml/min. The duration of one reaction cycle ranged from 2 to 8 h. After the finish of reaction cycle the reaction medium was removed, and the biocatalyst was rinsed with distilled water and a corresponding buffer. For freshly prepared biocatalysts, the total rate of the reaction (W_Σ) and the rate in medium (W_{medium}) were measured. For the determination of W_{medium}, an aliquot of the reaction medium was taken from the mixer after 1 h of circulation through the biocatalyst bed, and then was kept for an additional 1 h at 70 °C in the absence of the biocatalyst until the completion of the 2-h reaction cycle. The rate of the reaction in the presence of the heterogeneous biocatalyst (W_{cat}) was calculated as residual: $W_{cat} = W_\Sigma - W_{medium}$.

The initial activity of the biocatalyst (A_0) was estimated in terms of the total rate of the reaction in the first reaction cycle as W_Σ/g. After the biocatalyst underwent conditioning, and $W_{medium} \approx 0$, the steady-state activity was calculated as $A_{st} = W_{cat}$/g. Obviously, that the steady-state activity is the significant and the most important characteristic from a practical point of view, because it defines the total productivity of the biocatalyst approximately estimated as multiplication of $A_{st} \times 2\, t_{½}$.

The continuous process of isomerization of monosaccharides (glucose and fructose) was carried out in a plug flow reactor with a fixed bed of biocatalyst granules 1–4 mm in size and inert filler, glass balls 2 mm in diameter, the ratio of their volumes was 1:1. The use of the filler significantly reduces the hydrodynamic resistance of the bed. The reactor was placed into the thermostat with a temperature of $62 \pm 2\,^{\circ}C$, and a 3 M fructose solution was run through the fixed bed with a volume of 10 cm^3 from top to bottom at a flow rate of 0.02 ml/min. Aliquots were taken at the output of the reactor at regular intervals (once per day) and glucose concentrations were analyzed. The conversion of the substrate was calculated, and half-life time of biocatalyst ($t_{½}$) was determined.

The stability of the biocatalysts was examined in both the periodical and continuous regimes, and the inactivation was characterized in terms of half-life time of biocatalyst ($t_{½}$). In addition, we showed that the biocatalysts did not lose activity during their storage (for 18–20 h) in a buffer at ambient temperature between reaction cycles of the periodic process.

2.2.4. Measurements of physicochemical parameters

The specific surface ($S_{sp\ BET}$) was determined by thermal desorption of argon using a SORBI-M instrument (ZAO Meta, Russia). The size distribution of pores was estimated by mercury and nitrogen porosimetry using an AUTO-PORE 9200 and ASAP 2400 V3.07 instruments (Micromeritics, USA). The content of carbon in material (a) was found by thermal analysis using an STA-449 C Jupiter instrument (Netzsch, Germany). The electron-microscopic studies of the cleavages of composite biocatalysts were performed using scanning electron microscopes (SEM) JSM 6460 LV (JEOL, Japan) and LEO 1430 (LEO, Germany). Marks in the SEM images corresponded to distances in μm.

3. Results and discussion

3.1. Common peculiarities of preparing the biocatalysts

The optimization of the multi-component composition of the biocatalysts was performed simultaneously over two parameters, that is, a maximum value of the enzymatic activity and a high stability of granules in the aqueous reaction medium with pH 5–7. It is of interest that the content of biomass after optimization over both parameters was found to be dependent upon the taxonomy of microorganisms. Hence, the optimal content of biomass inside biocatalysts was found to be 10–15 wt% and 35–40 wt% for wild strain-producer *Arthrobacter nicotianae* and for recombinant strain-producer *E. coli*, respectively [13,21], as well as ~50% and 60–70% for *Rhococcus ruber* and for baker's yeast, respectively. Upon further increase of the biomass content, granules partially destructed to tiny pieces under reaction conditions, which mean that the biocatalysts' stability was very low. The content of nanocarbons should be no greater than 25 wt%. Also, it was revealed that the amount of SiO_2 as a binding agent should be not less than 20 wt%. A decrease of SiO_2 and an increase in the carbons or biomass contents also provided a damage of biocatalysts' granules under reaction conditions. The granules of the biocatalysts were also found to destruct quickly if the carbon material itself cannot be compressed with the formation of pellets at an high pressure. This means that the nanocarbon material was not a structure-forming (binding) agent.

The study of the texture of the biocatalysts prepared was carried out. The values of specific surface area ($S_{sp\ BET}$) for biocatalyst prepared by immuring of *A. nicotianae* and rec-*E.coli*, as well as yeast autolysates inside SiO_2–xerogel are ~180 m^2/g, ~70 m^2/g and ~90 m^2/g, respectively ($S_{sp} \approx 260\ m^2$/g for SiO_2–xerogel only). Mesopores with 10–20 nm diameters were found to predominate in the pore structures of the biocatalysts prepared; and they occupied up to 50% of their total pore volume. There were no micropores smaller than 3 nm in the pore structure of the biocatalysts. Due to mesoporous texture the biocatalysts were found to operate in kinetics region, the diffusion did not limit the rate of biocatalytic process. The values of $S_{sp\ BET}$ of biocatalyst prepared by immuring of yeast autolysates inside both SiO_2–xerogel and {nanocarbon-in-silica} composites are listed in Table 1. On the assumption of mechanical mixture of nanocarbons and silica the values of $S_{sp\ BET}$ of the composite biocatalysts have to increase by ~20–70 m^2/g. But in the majority of instances the S_{sp} of biocatalysts decrease after inclusion of nanocarbon inside SiO_2–xerogel (Table 1).

3.2. The conditioning freshly prepared biocatalysts to steady state

The conditioning of the freshly prepared biocatalysts was observed for 1–2 h. For the biocatalysts with glucose isomerase activity, the reaction rate decreased by 80–90% before following reaction cycles in periodic regime (Fig. 1a); in some cases, e.g. at a high content of microbial biomass inside the biocatalyst, $W_\Sigma \approx W_{medium}$. For the biocatalysts with invertase activity, the reaction rate decreased by 40–50% (Fig. 1b). The biocatalysts then work in quite a stable manner for a long-time period with a steady-state activity (A_{st}). Hence, the typical inactivation curve for the biocatalysts (Fig. 1) can be divided into two segments with different inactivation constants, k_{in}: the segment, in which an abrupt drop in the rate of the reaction is observed, and the second segment with steady-state activity. In the second segment, the kinetics was described by first-order exponential decay. Interestingly, the value of k_{in} for the biocatalysts prepared with the recombinant strain-producer, $3.5 \times 10^{-2}\ h^{-1}$ (70 °C), calculated in second segment of kinetic curve (Fig. 1a), was approximately equal to the k_{in} for the biocatalysts prepared with wild strain-producer, $3.8 \times 10^{-2}\ h^{-1}$ (70 °C), determined on kinetic curve of inactivation of these biocatalysts [22]. It has been known that glucose isomerase produced by wild strain *A. nicotianae* is intracellular enzyme which is located near cell wall and bond with the murein carcass; in fact, this enzyme is immobilized inside bacterial cells [23]. Recombinant strain *E. coli* produces glucose isomerase in the amount of ~50% of total intracellular proteins [24,25]. The agreement of values of k_{in} may be indirect evidence that the steady-state activity of the prepared biocatalysts was specified by glucose isomerase bond (immobilized) inside both wild and recombinant strain. During preparation of the biocatalysts (drying, compression) the bacteria were partially disrupted. As a result, produced by rec-*E.coli* glucose isomerase can be liberated from the recombinant cells to the silica matrix and then to the reaction medium. The very high value of reaction rate in the medium after removal of biocatalyst, often $W_\Sigma \approx W_{medium}$ in the first reaction cycle, was observed. The leaching of the enzymatic active substance from the biocatalyst caused the dramatic fall in the activity of the biocatalysts (Fig. 1).

We conceived the idea to include an additional component, capable of adsorption/adhesion of enzymatic active substances, into the composition of the biocatalysts in order to retain it inside the silica matrix. Previously it was demonstrated that carbon nanofibers (CNFs) can effectively adsorb proteins and enzymes [12]. In current work, it was found that double-wall carbon nanotubes

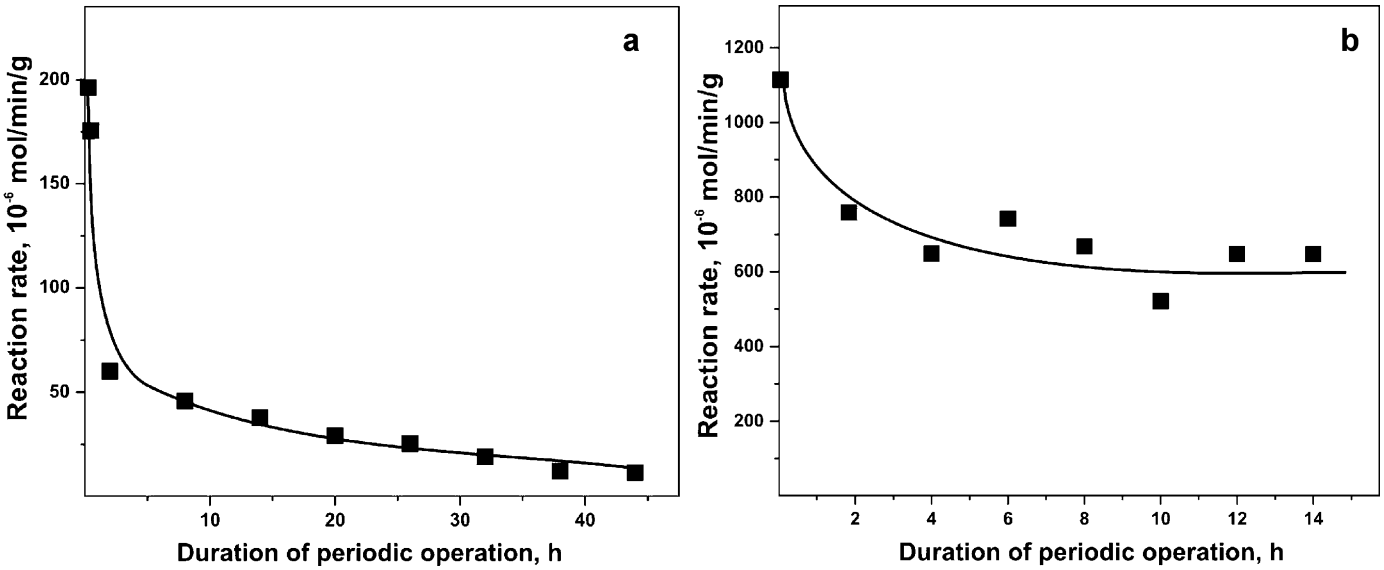

Fig. 1. The decrease of reaction rate owing to conditioning biocatalysts under reaction conditions: a – the biocatalysts prepared by immuring bacterial cells of recombinant strain-producer of *E.coli*, b – the biocatalysts prepared by immuring baker's yeast autolysates.

(DWCNTs) completely extracted albumin from the solutions; the adsorption value was estimated as 19–22 mg/g.

3.3. Biocatalysts with glucose isomerase activity

As mentioned above, a dramatic fall in the initial biocatalysts' GI activity during conditioning was observed (Fig. 1a). We supposed that the leaching of the enzymatic proteins (not whole bacterial cells or their compartments) from SiO_2–xerogel occurred. At first, the initial A_0 was in several times higher than A_{st}, such, ≥500 and ~100 U/g, respectively. If the downfall may be arranged by properly complete leaching biomass from biocatalyst, we may estimate the turbidity of the reaction medium with observed W_{medium}. In this case, the optical density would be detectable (more than 0.4 at 590 nm), whereas the reaction medium remained transparent for the hours upon biocatalytic process proceeding. Then, the foaming of transparent reaction medium (look like in protein solutions) was observed.

To select efficient adsorbent for glucose isomerase for inclusion inside biocatalysts we take into account both the chemical origin of the protein molecule and the supports. Previously in [26] we demonstrated the important role of mutual correspondence of hydrophobic-hydrophilic characters between enzyme molecules, in particular the active site, and support surface for preparing active and highly stable biocatalysts. It was found in [25,27] that the glucose isomerase molecule consists of 73.2% hydrophobic and 26.8% hydrophilic amino acids; and this fact means that the enzyme exhibits pronounced hydrophobic properties. On the other hand, the carbonic supports were predominately hydrophobic. We conceived the idea to include carbons into the composite biocatalysts in order to retain enzyme inside SiO_2–xerogel due to hydrophobic–hydrophobic interactions and, as a result, to increase the steady-state activity of the biocatalysts. In the electron microscopic images of the cleavages of the composite biocatalysts, one can see that both the cells of rec-*E.coli* and CNTs are immured in the structure of silica xerogel (Fig. 2). But it has been found experimentally that the inclusion of nanocarbons into the composition of the biocatalysts barely influenced on their biocatalytic properties. Thus, the systematic deviations in the A_{st} values for the composite biocatalysts in comparison with the biocatalysts not containing nanocarbons were 10–15% in the direction of increasing the measuring parameters. The value of the steady-state biocatalyst's activity was ~100 U/g. "Dry" cross-linking was performed in order to increase the stability of the biocatalysts. Glutaric dialdehyde (GA) was found to be strong inhibitor for glucose isomerase; enzyme completely loses the activity at GA concentration more than 2 wt%. This is most likely due to the interaction of aldehyde groups with NH_2-groups of the amino acids involved into the active

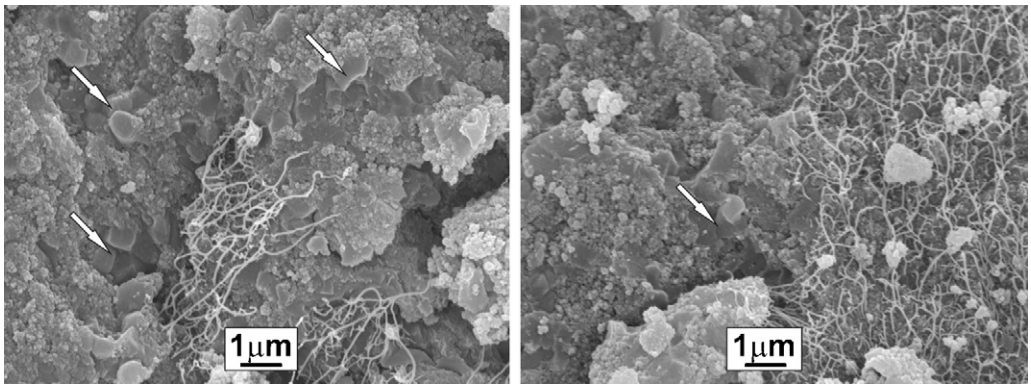

Fig. 2. Electron-microscopic images of cleavages of the biocatalyst prepared by immuring rec-*E.coli* inside {DWCNTs-in-silica} composite. Arrows indicate immured bacterial cells.

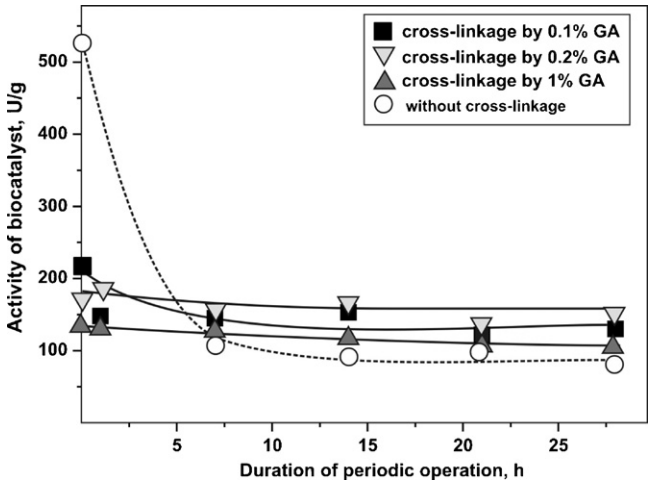

Fig. 3. The enzyme activity of the biocatalysts prepared by immuring rec-*E.coli* inside {CNFs-in-silica} composite in dependence on concentration of glutaric dialdehide used for "dry" cross-linking.

site of the enzyme and directly into biocatalysis. Actually, two residues of asparagine, Asp-104 and Asp-339 are in active site of GI [25]. During "dry" cross-linking by glutaric dialdehyde at concentration 0.1–1 wt%, the initial activity of the biocatalysts decreased by a factor of 3–5; and no abrupt drop in the rate of the reaction was observed, $A_0 \approx A_{st} \approx 110–150$ U/g (Fig. 3). We hope that the nanocarbons included will effect on the degree of inactivation of glucose isomerase by GA. But it has been found experimentally that nanocarbons did not have a protective effect. "Dry" cross-linking allowed the steady-state activity to be increased by a factor of ~1.5 in comparison to the biocatalyst not undergoing cross-linking, and by a factor of ~2 as compared to "wet" cross-linking, described in [13]. Thus, at the same content of glutaric dialdehyde calculated per 1 g of dry cells (140–160 mg GA/g), the steady-state activity of the biocatalysts was ~150 U/g and ~80 U/g, respectively. "Dry" cross-linking was preferable treatment in comparison with "wet" cross-linking, since the concentration of the inhibitor (GA) and its uniform distribution in the biocatalyst bulk was more precisely controlled.

To elucidate the non-considerable effects observed upon inclusion of nanocarbons, we carried out the study on the ability of the glucose isomerase itself to be adsorbed on various solid supports. This property of the enzyme has not previously been subject to detailed study; and it was believed that the use of a purified enzyme is reasonable for the preparing heterogeneous biocatalysts by adsorption, since it has a higher specific glucose isomerase activity in comparison to the suspended bacterial cells. Again we examined the chemical origin of enzyme molecules and the supports studied. As was noted above, the glucose isomerase from *A. nicotianae* contains 73.2% hydrophobic and 26.8% hydrophilic amino acids; and 54.7% hydrophilic acids have a negative charge, and 45.3% are positively charged [24,25]. Consequently, the isoelectric point (pI) of this enzyme lies in the acidic range, and at neutral pH values, the molecule has a slight total negative charge. Mesoporous supports such as θ-alumina ($S_{sp} = 55\ m^2/g$), silica (SiO_2, type Silochrome®, $S_{sp} = 66\ m^2/g$), carbonized γ-alumina ($S_{sp} = 220\ m^2/g$), and carbon support (type Sibunit®, $S_{sp} = 550\ m^2/g$) were used for the adsorptive immobilization of the glucose isomerase. It is well-known that at neutral pH the surface of Al_2O_3 is positively charged, the surface of SiO_2 and Sibunit has a slight negative charge; also carbon-containing supports exhibit hydrophobic properties. Glucose isomerase for study was partially purified from wild strain-producer *A. nicotianae* [28] and recombinant *E. coli* [24]. The results on adsorptive immobilization described in [22] suggested, that the crucial factor for adsorption of glucose isomerase was not the chemical origin and electrical charge of the supports, but the accessible surface area, S_{acces}. The S_{acces} value was estimated from pore-distribution diagrams under the true assumption that the pores, the sizes of which are more than diameter of enzyme molecules, are accessible for their transport and adsorption. A glucose isomerase molecule from *A. nicotianae* (MW = 190 kDa) is a tetramer consisting of 4 identical subunits [25]; the diameter of the enzyme molecule in aqua surrounding is more than 15 nm. Therefore, the pores in diameter more than 20 nm are exactly accessible for transfer and binding enzyme inside porous supports' area. It was found that the larger S_{acces}, the greater the adsorption value of the protein. Thus, the maximum adsorption was observed on SiO_2 ($S_{sp} \approx S_{acces} = 60\ m^2/g$); the adsorption values were 13 and 27 mg/g for *A. nicotianae* and rec-*E.coli*, respectively. And vice versa, the minimum adsorption was observed on carbonized γ-Al_2O_3 ($S_{acces} = 7\ m^2/g$); the adsorption values were 4 and 9 mg/g for *A. nicotianae* and rec-*E.coli*, respectively. The amount of adsorbed GI was found to increase approximately linearly with the increase of S_{acces} at the average value of 0.2 mg of protein per 1 m^2 of accessible surface area. Considering this value and MW of enzyme, we can estimate the diameter of adsorbed GI molecule; and this diameter was approximately 30 nm and close to the one for hydrated enzyme molecules as mentioned above. Therefore, a dense monolayer of adsorbed GI molecules was formed on the surface of various solid supports. We estimate a selectivity of glucose isomerase adsorption by comparing the specific enzyme activity (A_{sp}, U/mg of protein) in solutions over the solid supports before adsorption and after finishing adsorption. For SiO_2 and carbon Sibunit, the specific GI activity at the finish was found to be 1.5–2.5 times higher than A_{sp} before adsorption. This may be arranged by preferable adsorption of protein impurities accompanying enzyme purification. For θ-Al_2O_3, the specific glucose isomerase activities before and after adsorption had close values, 17 and 16 U/mg of protein, respectively. Since, glucose isomerase and proteins impurities were equally adsorbed on the alumina surface.

In regards to activity of adsorbed glucose isomerase, the enzyme completely lost its activity during adsorption (more then 90% activity) if to compare with the activity of soluble enzyme. For carbon-containing supports, complete inactivation of the enzyme from *A. nicotianae* was observed. Perhaps, due to hydrophobic–hydrophobic interaction of enzyme molecule and carbon surface, an inaccurate orientation of the active site occurred; it was blocked by support and biocatalysis became impossible. Indeed, the residues of hydrophobic aminoacids (tryptophan Trp-188, Trp-130, and histidine His-101) are involved in active site of GI [29]. Actually, the mutual correspondence of enzyme-support hydrophobic–hydrophilic characters has a dominant role in retention of biocatalyst's activity as mentioned above [26].

It was found that the activities of the biocatalysts depended on the taxonomy of the strain-producer of the glucose isomerase, that is, the wild or recombinant strains were used for enzyme purification. Often we observed opposite behaviors. For example, the biocatalyst prepared by adsorption of glucose isomerase from *A. nicotianae* on SiO_2 exhibited the maximum initial activity, $A_0 = 17$ U/g of biocatalyst, owing to the highest values of both adsorption and specific activity retained. On the contrary, the biocatalyst prepared by adsorption of glucose isomerase from rec-*E.coli* on SiO_2 had a minimum initial activity, $A_0 = 2$ U/g, although the adsorption was relatively high (27 mg/g). Or, as mentioned above, GI from *A. nicotianae* completely inactivated on carbonized γ-alumina, while the biocatalyst based on GI from rec-*E.coli* had a comparatively high initial activity (16 U/g). Probably, such differences were determined by the nature of protein impurities and non-specific adsorption of GI in the presence of these impurities.

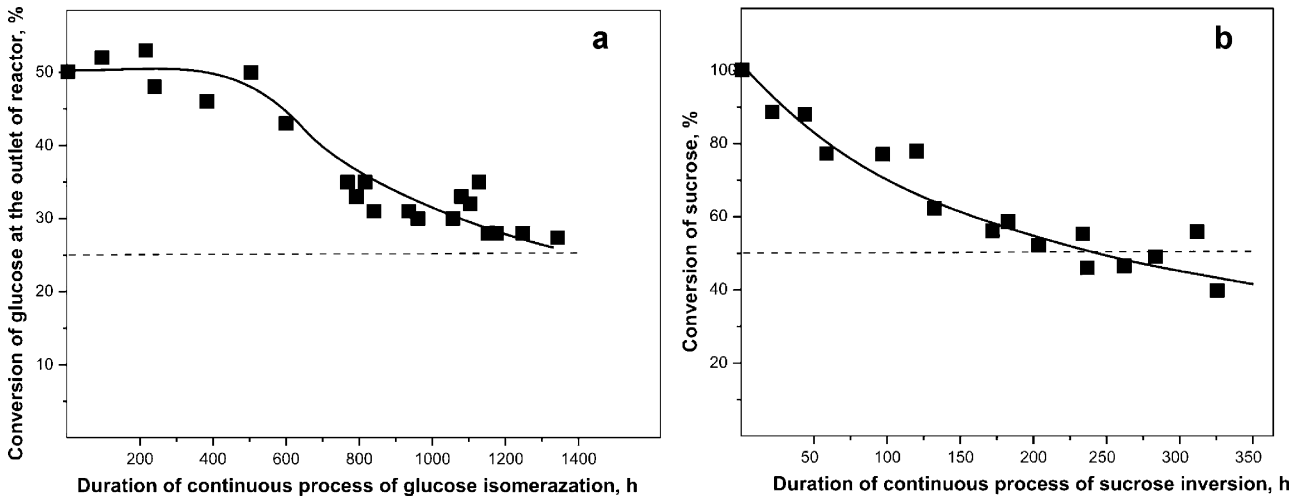

Fig. 4. The testing of operational stability of the biocatalysts: a – the biocatalysts prepared by immuring rec-*E.coli*, b – the biocatalysts prepared by immuring baker's yeast autolysates, at 62 ± 2 °C and 50 °C, respectively.

In regards to stability of adsorbed glucose isomerase, the stability of the biocatalysts was extremely low. The maximum half-time of inactivation ($t_{½}$) observed for the biocatalysts prepared by adsorption of the enzyme from *A. nicotianae* on θ-Al_2O_3 was not more than 8 h (often $t_{½} \approx 2$ h). The main reason of biocatalysts' inactivation was shown to be desorption of the enzyme from the support's surface. As such, for GI on SiO_2, the rate of the reaction in the medium, W_{medium}, was two times higher than W_{cat} in the first reaction cycle ($t_{½} < 2$ h). This was evident that the adsorption was very weak perhaps due to the same electrical negative charges of molecule and support's silica surface as mentioned above. On the whole, it was very hard to find any correlation between the ability of partially purified GI to be adsorbed, as well as supports' surface character and properties (activity and stability) of the biocatalysts prepared. But we can make a conclusion that the biocatalysts prepared by adsorption of partially purified glucose isomerase on solid supports exhibit very poor activity and stability. Now we can explain the non considerable effects observed upon inclusion nanocarbons inside composite biocatalysts prepared, because we take into consideration the peculiarities inherent for the glucose isomerase, that is, nonspecific and weak adsorption, and a complete loss (90–100%) of enzyme activity in the adsorbed state on any surfaces, in particular carbons.

Finally, returning to the multi-component biocatalysts with glucose isomerase activity, we conclude that the maximal effect on increase of steady-state activity and the stability of the biocatalysts based on {nanocarbon-in-silica} composites did not exceed 1.2 times in comparison to the biocatalysts prepared without nanocarbons. It was found that $t_{½}$ of biocatalysts prepared by immuring of rec-*E.coli* inside SiO_2–xerogel (with nanocarbon or not) exceeded 1400 h for continuous process of glucose isomerization at 62 ± 2 °C (Fig. 4a).

3.4. Biocatalysts with invertase activity

A further study of the composite biocatalysts was performed using enzymatic active substances capable to be adsorbed on nanocarbons; and the preliminary study was carried out. As mentioned above, there are experimental difficulties for operating with fine carbon powders in aqueous solutions, concerning sedimentation, filtration, analysis of solutions. Therefore, we prepared granules of nanocarbons by compression of fine powder into pellets and fractionation into granules 0.1–0.2 mm in size. Notice that some of the nanocarbons such as multi-walled CNTs and onion-like nanocarbon did not form pellets; and granules of nanodiamonds immediately destructed into tiny pieces at contact with an aqueous solution.

Previously, it was shown that invertase activity of yeast autolysates was fully retained after adhesion on various solid supports [14]. Because if this, we can estimate the amount and tightness of adhesion of yeast autolysates via comparison of the initial and steady-state invertase activity of the biocatalysts prepared. It was more accurate than the measuring by decrease of optical density of the suspensions before and after adhesion; often the turbidity of suspensions increased after contacting with nanocarbons. It was found that yeast autolysates were not adhering on granules of DWCNTs. For MWoCNTs and CNFs, the total amounts of adhered autolysates were found to be ~5 mg/g and ~2 mg/g, respectively. Also, 73% and 38% of total amount were adhered very tightly on MWoCNTs and CNFs, respectively; and the steady-state invertase activities were equal to 103 U and 20 U per 1 g of granulated MWoCNTs and CNFs, respectively. Hence, due to tight adhesion the MWoCNTs was the best adsorbent for yeast autolysates. Now we try to find the correlation between the tightness of autolysates' adhesion on the nanocarbons and the steady-state activity of the composite biocatalysts prepared.

The properties (invertase activity and stability) of the composite biocatalysts were investigated systematically. It was found that an increase in the content of included CNFs (adhesion occurred on CNFs as described above) by a factor of 5 (from 5% to 25%) provided an increase in the steady-state activity of the biocatalysts by a factor of 1.8. As the data of Table 1 suggested, the activity of the biocatalysts containing DWCNTs (adhesion did not occured on DWCNTs as described above) insignificantly differed from the activity of the biocatalysts prepared without carbon (Table 1). A comparison of the data in Table 1 and Fig. 5 indicated that the steady-state activity of the prepared biocatalysts was defined by the adsorption ability of the nanocarbons; and the more efficient the adhesion of yeast autolysates (on MWoCNTs as described above) the higher was A_{st}. As the data of Table 1 suggested, the inclusion of MWCNTs inside SiO_2–xerogel caused the maximal effect on biocatalytic properties of immured autolysates; the value of steady-state activity was 5–6-fold higher than one for SiO_2–xerogel only. In addition, the parameter describing the efficiency of immobilization was calculated as a ratio (in %) of the A_{st} to a theoretically possible maximum value, the latter was estimated using the content of the biomass inside the biocatalyst and the activity of suspended autolysates. Obviously, a decrease of activity occurred during preparation

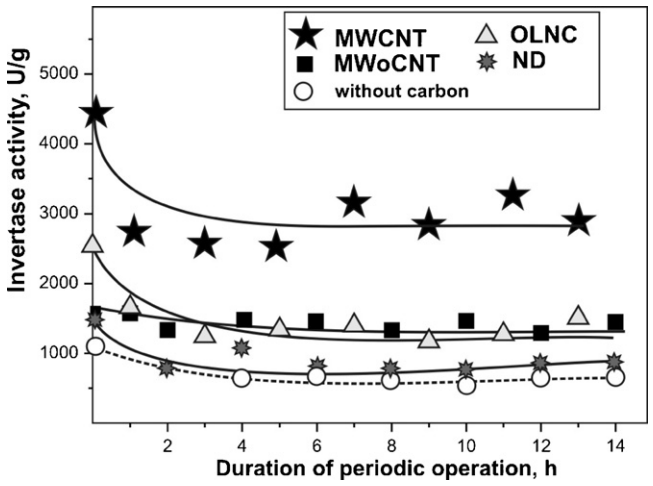

Fig. 5. The enzyme activity of the biocatalysts prepared by immuring baker's yeast autolysates inside {nanocarbon-in-silica} composites in dependence on type of nanocarbon.

of the biocatalysts (upon drying and pressing). In the majority of instances, only 7–9% of activity of suspended autolysates was retained during preparing of composite biocatalysts, but for MWCNTs this value run up to 20%. "Dry" cross-linking of composite biocatalysts by glutaric dialdehyde (GA) was performed in order to increase their stability. Negligible effect on biocatalytic properties was observed at concentration GA up to 10 wt% (by contrast with glucose isomerase, GA at concentration >1 wt% completely inactivate GI).

Using the data listed in Table 1 as a framework, we can estimate the adsorption ability of those nanocarbons that we could not compress into granules and determine their adsorption ability directly. For example, nanodiamonds did not adsorb yeast autolysates, since the biocatalytic properties did not change upon the inclusion of this component inside the biocatalysts (in a similar manner, DWCNTs do not adsorb yeast autolysates as described above). In contrast, MWCNTs were the most effective adsorbents with respect to yeast autolysates, since the steady-state activity of the biocatalysts has a maximum value of ~3000 U/g (Fig. 5). On the basis of data presented in Table 1 and Fig. 5, the nanocarbons can be arranged in following order with respect to adsorb the enzymatic active substances, in particular yeast autolysates: MWCNTs (320 m^2/g) ≫ MWoCNTs (330 m^2/g) > OLNC (485 m^2/g) > CNF (162 m^2/g) > DWCNTs (95 m^2/g) ≈ ND (325 m^2/g). For carbons with filamentous nanostructure, a correlation of the adsorption ability with specific surface area can be observed. In the electron microscopic images of biocatalyst cleavages, smooth areas were arranged by yeast autolysates (Fig. 6,). Carbon nanotubes (Fig. 6a) and multi-walled carbon nanotubes (Fig. 6b) were the most clearly visible. Carbon nanofibers are observed in the form of short pieces of CNF cracked (Fig. 6c). The presence of onion-like nanocarbon was confirmed by an increase in the roughness of the yeast autolysate smooth areas (Fig. 6d).

Finally, returning to the multi-component biocatalysts with invertase activity, we conclude that the maximal positive effect on the biocatalytic properties was observed for the biocatalysts based on {MWCNTs-in-silica} composites. We reached the 6-fold increase of steady-state activity in comparison with the silica-based biocatalysts; and multi-walled carbon nanotubes were the most effective adsorbents for enzymatic active substances. It was found that $t_{½}$ of biocatalysts with invertase activity ca. 1000 U/g prepared by immuring of bakers' yeast autolysates inside {CNFs-in-silica} composite was approximately equal to 250 h for continuous process of sucrose inversion at 50 °C (Fig. 4b).

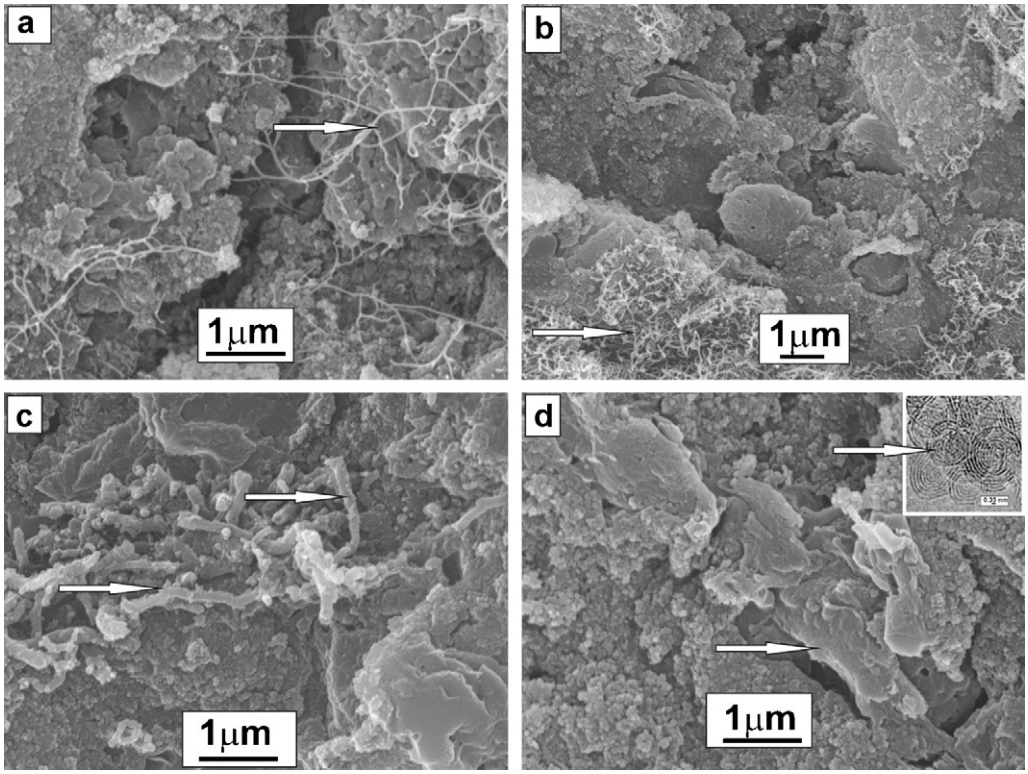

Fig. 6. Electron-microscopic images of cleavages of the biocatalyst prepared by immuring baker's yeast autolysates inside {nanocarbon-in-silica} composites: a – {DWCNTs-in-silica), b – {MWCNTs-in-silica}, c – {CNFs-in-silica}, d – {OLNC-in-silica}. Arrows indicate included nanocarbon material.

4. Conclusions

According to the comparative study of the biocatalytic properties (steady-state activity and stability) of the multi-component biocatalysts prepared by immuring of enzymatic active substances inside {nanorarbon-in-silica) composites, the inclusion of nanocarbons (nanotubes, nanofibers, and onion-like nanocarbon) was reasonable if the enzymatic active substances had a capability of being adsorbed on the carbonic material.

The glucose isomerase enzyme was weakly adsorbed on the solid supports; and that was a reason for non-considerable effect of including the nanocarbons inside silica-based biocatalysts. The maximum increase of steady-state activity (by a factor of 1.5–2) was revealed during "dry" cross-linking of the biocatalysts prepared by immuring recombinant strain-producer *E. coli* inside the {nanorarbon-in-silica) composites via glutaric dialdehyde (0.1–1 wt%) treatment. The maximum value of the steady-state activity was 150–160 U/g at 70 °C, $t_{½} \geq 1400$ h under continuous glucose/fructose isomerization.

Yeast autolysates were capable of being tightly adhered on the nanocarbons; and an increase in the invertase steady-state activity value by a factor of 2–6 in comparison to SiO_2–xerogel (without carbon) was observed. Correlation between the adsorption ability of yeast autolysates on nanocarbons and the steady-state activity value of the biocatalysts was found. The highest value of the steady-state activity of the biocatalysts prepared using {MWCNTs-in silica} composite was ~3000 U/g at 50 °C, $t_{½} \geq 250$ h under continuous sucrose inversion.

From the experimental observations, the nanocarbons can be arranged in following order with respect to adsorb/adhere the enzymatic active substances, in particular yeast autolysates: MWCNTs (320 m^2/g) ≫ MWoCNTs (330 m^2/g) > OLNC (485 m^2/g) > CNF (162 m^2/g) > DWCNTs (95 m^2/g) ≈ ND (325 m^2/g). For carbons with filamentous nanostructure, a correlation of the adsorption ability with specific surface area was observed. The investigation in the area of application of nanocarbons in heterogeneous biocatalysis will be continued.

Acknowledgments

The authors are grateful to L.I. Sapunova, E.A. Shlyahotko and E. Flahaut who kindly donated the samples for the study.

References

[1] A.N. Shtykov, T. Yu., Rusanova Rus. Chem. J. 52 (2008) 92–100.
[2] T. Fukushima, A. Kosaka, Science 300 (2003) 2072–2074.
[3] R.J. Chen, S. Bangsaruntip, K.A. Drouvakalis, Proc. Natl. Acad. Sci. 100 (2003) 4984–4989.
[4] S. Yadav, R. Devi, S. Kumari, S. Yadav, C.S. Pundir, J. Biotechnol. 151 (2011) 212–217.
[5] H. Li, D.Q. Wang, B.L. Liu, L.Z. Gao, Colloid, Surf. B: Biointerfaces 33 (2004) 85–88.
[6] H.Y. Zhao, W. Zheng, Z.X. Meng, H.M. Zhou, X.X. Xu, Z. Li, Y.F. Zheng, Biosens. Bioelectron. 24 (2009) 2352–2357.
[7] R. Pauliukaite, M.E. Ghica, O. Fatibello-Filho, C.M. Brett, Anal. Chem. 81 (2009) 5364–5372.
[8] D.-J. Yun, K. Hong, S. Kim, W.-M. Yun, J. Jang, W.-S. Kwon, Ch-E. Park, Sh-W. Rhee, Appl. Mater. Interfaces 3 (2011) 43–49.
[9] J. Manso, M.L. Mena, P. Yanez-Sedeno, J. Pingarron, J. Electroanal. Chem. 603 (2007) 1–7.
[10] N.V. Shvedene, D.V. Chernyshov, I.V. Pletnev, Rus. Chem. J. 52 (2008) 80–91.
[11] B.R. Azamian, J.J. Davis, K.S. Coleman, C. Bagshaw, M.L.H. Green, J. Amer. Chem. Soc. 124 (2002) 12664–12665.
[12] G.A. Kovalenko, E.V. Kuznetsova, Yul. Mogilnykh, I.S. Andreeva, D.G. Kuvshinov, N.A. Rudina, Carbon 39 (2001) 1133–1143.
[13] G.A. Kovalenko, L.V. Perminova, V.M. Lenskaya, L.I. Sapunova, E.A. Shlyahotko, A.G. Lobanok, Appl. Biochem. Microbiol. 47 (2011) 151–157.
[14] G.A. Kovalenko, L.V. Perminova, G.V. Plaksin, O.V. Komova, T.V. Chuenko, N.A.Rudina, Appl. Biochem. Microbiol. 41 (2005) 399–403.
[15] E. Flahaut, R. Bacsa, A. Peigney, Ch. Laurent, Chem. Commun. (2003) 1442–1443.
[16] A.N. Usoltseva, V.L.Kuznetsov, N.A. Rudina, E.M. Moroz, M. Haluska, S. Roth, Phys. Status Solidi (b) 244 (2007) 3920–3924.
[17] T. Kyotani, S. Nakazaki, W.-H. Xu, A. Tomita, Carbon 39 (2001) 782–785.
[18] V.L. Kuznetsov, A.L. Chuvilin, Yu V. Butenko, I. Yu Malkov, V.M. Titov, Chem. Phys. Lett. 222 (1994) 343–348.
[19] G.G. Kuvshinov, Yu.F I., I.S. Mogilnykh, D.G. Kuvshinov, V.I. Zaikovskii, L.B. Avdeeva, Carbon 36 (1998) 87–97.
[20] L.G. Simonova, V.B. Fenelonov, V.L. Dzisko, G.N. Kryukova, B.P. Shmachkova, Kinet. Catal. 23 (1982) 138–143.
[21] L.V. Perminova, G.A. Kovalenko, N.A. Rudina, L.I. Sapunova, I.O. Tamkovich, A.G. Lobanok, Appl. Biochem. Microbiol. 45 (2009) 389–394.
[22] L.V. Perminova, G.A. Kovalenko, T.V. Chuenko, N.A. Rudina, Kinet. Catal. 53 (2011) 145–153.
[23] L.I. Sapunova, A.G. Lobanok, E.V. Parahnya, I.O Kazakevitch, Biotechnology (Rus.) 5 (2000) 66–71.
[24] E.A. Shlyahotko, L.I. Sapunova, A.G. Lobanok, A.N. Evtushenkov, Dokl. Natl. Acad. Belarus 50 (2006) 73–76.
[25] E.A. Shlyahotko, L.I. Sapunova, A.G. Lobanok, A.M. Korzhik, A.N. Evtushenkov, Dokl. Natl. Acad. Belarus 51 (2007) 52–56.
[26] V.D.Sokolovskii, G.A.Kovalenko, Biotechnol. Bioeng. 32 (1988) 916–919.
[27] M. Meng, M. Bagdasarian, J.G. Zeikus, Proc. Natl. Acad. Sci. 90 (1993) 8459–8463.
[28] E.V. Parahnya, L.I. Sapunova, A.G. Lobanok, I.O Kazakevitch, Biotechnology (Rus.) 5 (1999) 40–46.
[29] K. Henrick, C.A. Collyer, D.M. Blow, J. Mol. Biol. 208 (1989) 129–157.