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Carbon–Silica Composite Matrices for Preparing Heterogeneous Biocatalysts with Glucose Isomerase Activity

L. V. Perminova[a], G. A. Kovalenko[a, b], T. V. Chuenko[a], and N. A. Rudina[a]

[a] *Boreskov Institute of Catalysis, Siberian Branch, Russian Academy of Sciences, Novosibirsk, 630090 Russia*
e-mail: galina@catalysis.ru

[b] *Novosibirsk State University, Novosibirsk, 630090 Russia*

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Abstract—Comparative studies have been carried out on the preparation of carbon–silica composite matrices for heterogeneous biocatalysts with glucose isomerase activity. Carbon nanotubes and nanofibers have been included inside SiO_2 xerogel. The enzymatic activity and operating stability of the biocatalysts have been investigated. Bacterial cells of a recombinant glucose isomerase producer strain have been used as the enzymatically active component of the biocatalysts. The steady-state activity of the biocatalysts subjected to "dry" cross linking with a glutaraldehyde solution (0.1–1%) is 1.5 times higher than the activity of the biocatalysts containing no nanocarbon. The initial and steady-state glucose-isomerase activities of the biocatalysts at 70°C are ~520–540 and ~150–160 μmol min^{-1} g^{-1}, respectively. The half-inactivation time of the biocatalysts under continuous monosaccharide (glucose, fructose) isomerization conditions is up to ~1500 h.

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Preparation and use of various composites containing nanostructured carbon (nanotubes, nanofibers, nanoonions) is a line of advance of contemporary science and technology and is widely discussed in the popular scientific literature. Here, we present the pioneering study in the synthesis of nanocarbon–silica composites and their use in heterogeneous biocatalysis, for example, in the preparation of biocatalysts with glucose isomerase (GI) activity. These biocatalysts are employed in the isomerization of monosaccharides (glucose or fructose) into glucose–fructose syrup (GFS), the natural substitute for cane and beet sugar. Although biocatalysts with GI activity are presently produced on a large scale (~1500 t per year [1–3]), biocatalyst manufacturers are performing extensive studies aimed at improving the existing biocatalysts and at developing new ones.

It is interesting that the time interval between the discovery of glucose isomerase in bacteria (1957) and the implementation of the first commercial process using this enzyme in the heterogeneous (immobilized) state (1973) is shorter than 20 years. This extraordinarily quick process commercialization is due to a concurrence of circumstances, specifically, the default of cane sugar export to the United States [3]. Twelve manufacturers using these biocatalysts operated in the market in the 1980s, and only five companies did so in the 2000s [1]. The biggest supplier of biocatalysts with GI activity is Novozymes Co. (Denmark), which manufactures 42% of the total output of these biocatalysts (Fig. 1). Each manufacturer has its own biocatalyst preparation technology based on the immobilization of some enzymatically active component, namely, the enzyme itself (CPC, Genencor, and UOP Cos.), cell homogenate (Novozymes), or whole cells (Nagase) [1–3]. The commercial biocatalysts meet the following requirements: in the continuous isomerization of 40–47 wt % glucose syrup, the half-inactivation time ($t_{1/2}$) is 1200–1800 h at 60°C and the total productivity is 1–4 t of GFS on a dry basis per kilogram of biocatalyst. Novozymes has recently announced a new biocatalyst with a record productivity of 18 (t GFS)/(kg biocatalyst). The biocatalyst is placed into a columnar reactor ~5 m in height to make a fixed bed 8–9 m^3 in volume. Glucose syrup is fed from the top down at a rate of 1–6 bed volumes per hour, depending on the activity of the biocatalyst.

Of interest is the dynamics of improvement of commercial biocatalysts with GI activity (e.g., in Novozymes Co.) [4, 5]. The first GI-active product, produced in 1973, was flakes obtained by spray drying of a soluble enzyme partially isolated by thawing frozen biomass. This biocatalyst was very expensive and was used in GFS production over a very short time. The first-generation biocatalyst Sweetzyme A, which appeared in 1974, was produced by cross-linking of enzymatically active *Bacillus coagulans* cell biomass with glutaraldehyde (GA). The resulting gelatinous mixture dried and milled into 0.1–0.35 mm particles, which were then used in a batch reactor. This biocatalyst had serious drawbacks: its granules wore out rapidly, and the biocatalyst had unsatisfactory thermal stability at 60°C, which had to be enhanced by adding Co^{2+} salts. The second-generation biocatalysts—

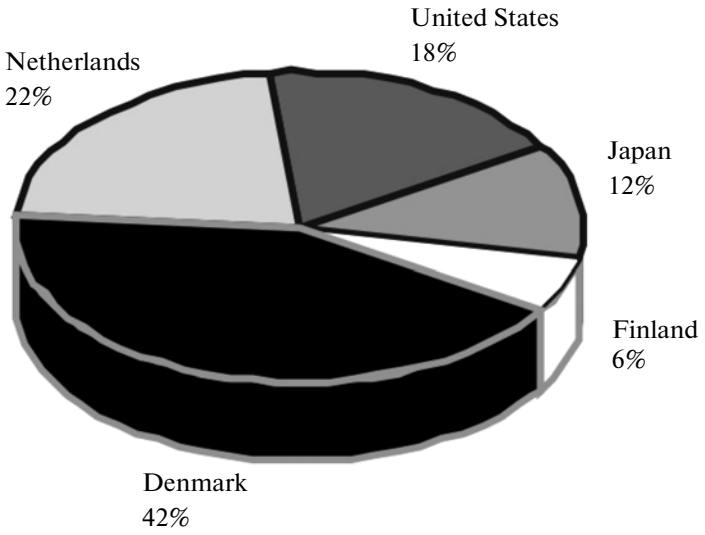

Fig. 1. Biggest manufacturers of heterogeneous biocatalysts with glucose isomerase activity and their contributions (%) to the world output of these products.

Sweetzyme E and Sweetzyme H—were produced by extrusion, through a draw plate with a hole diameter of 1 mm, of the plastic mixture obtained by cross-linking of microbial biomass with GA. The extrudate was cut into short cylindrical pellets, which were then dried and were used in a flow fixed-bed reactor. Alumina powder was added to the biocatalyst in order to improve the hydrodynamic properties of the biocatalyst bed. The third-generation biocatalysts—Sweetzyme S, Sweetzyme Q, and Sweetzyme T—were produced by cross-linking of homogenates of destroyed *B. coagulans* and *Streptomyces murinus* cells followed by extrusion and drying. Magnesium oxide powder and glucose were often added to the mass to be extruded. Glucose dissolved in the reaction medium, increasing the porosity of the biocatalyst, and this diminished the diffusion limitations. It was recommended that the catalyst be conditioned before use by soaking/wetting it in glucose syrup or GFS. This increased the size of the pellets by a factor of 2. Obviously, third-generation biocatalysts are presently used in the continuous glucose isomerization process in GFC production. As for Russian research in this area, Biotekhnologiya Co. was developing a GI-active catalyst (Imfruzym) in the 1980s, keeping pace with foreign researchers, and the All-Union Institute of Starch Products was developing a GFS production technology [5]. The Imfruzym biocatalyst was produced by cross-linking of *Actinomyces albogriseolis* biomass with gelatin. Its activity was 200–250 U/g, and its total productivity was 1.5 t/(kg biocatalyst) [5].

Owing to the advances in molecular biology and genetic engineering methods of making recombinant GI superproducer strains, it is possible to predict the improvements that will be made to the biocatalysts. It is likely that the next-generation biocatalysts will be prepared using either recombinant microorganisms or an enzyme isolated from these strains, in which the desired enzyme (GI) accounts for 15–60% of the total amount of intracellular proteins synthesized by the cell [3]. These biocatalysts will likely give way to ones that will be prepared using modified recombinant enzymes, such as GI with six terminal histidine residues (His_6-GI), whose presence allows the high-purity enzyme to be isolated in a single step on Ni-containing chromatographic supports [6].

The *xylA* gene, which encodes the enzymatic protein GI, was isolated, and its structure was determined [7, 8]. Genetic engineering works on cloning of the *xylA* gene resulted in construction of the recombinant producer strain *Escherichia coli* BL21(DE3)/*pET24bxylA* (rec-*E. coli*), in which GI accounts for ~50% of the intercellular protein [7, 8]. There have been studies on heterogeneous biocatalysts with GI activity prepared using the natural producer strain of *Arthrobacter nicotianae* (*xylA* gene donor) and the recombinant strain of *E. coli* (*xylA* gene recipient) by incorporation of microbial biomass into a SiO_2 xerogel matrix [9–11].

The purpose of this study was to obtain nanocarbon–silicate composites with the recombinant producer strain of *E. coli* included inside this, to prepare composite heterogeneous biocatalysts with GI activity, and to compare the enzymatic activity and stability of biocatalysts differing in carbon–silicate matrix composition and in conditions under which cross-linking with GA was performed.

EXPERIMENTAL

The recombinant strain *E. coli* BL21(DE3)/*pET24bxylA* was used, for which the GI gene induc-

tion, enzymatic protein expression, and conditions of submerged culturing were described in detail [7, 8, 11].

Heterogeneous biocatalysts were prepared by including microbial biomass inside carbon–silica composites in the same way as in our earlier studies [9–11]. Along with the main components—biomass (*1*) + SiO_2 (*2*) + Co_xO_y (*3*)—a nanocarbon-containing component (*4*) was introduced in the mixture. The proportion of the nanocarbon component did not exceed 15 wt %, because the biocatalyst granules containing more than 20% nanocarbon lost their stability and disintegrated during their operation in the buffer reaction medium. The resulting wet uniform mixture (*1* + *2* + *3* + *4*) was air-dried, ground into fine powder, pressed into pellets at a surplus pressure of 150 atm, and fractionated to obtain dry heterogeneous biocatalyst granules 1–4 mm in size. Cross-linking was carried out by keeping the dry biocatalyst in a 0.1–1% GA solution for 4 h at 13–15°C (so-called dry cross-linking). The dry biocatalysts were treated with γ-aminopropyltriethoxysilane (γ-APTES) by keeping them in a toluene solution of γ-APTES for 24 h at 20–22°C. Next, the biocatalysts were washed with toluene, dried in air, and washed with a buffer solution at pH 7.0. Double inclusion of microbial biomass inside a SiO_2 xerogel was performed as follows. Dry biocatalyst granules were ground into a fine powder. The powder was combined with a silicon dioxide hydrogel (component *2*), and the mixture was pressed and granulated as was described above.

Various carbon-containing adsorbents studied in detail in earlier works [12–15] were included inside composite biocatalysts. These absorbents were the carbon support Sibunit [16], massive catalytic filamentous carbon (CFC) [17], carbonized sapropels [18], activated carbon, and graphite. It was demonstrated in these works that CFC-based mesoporous supports and Sibunit are the best adsorbents for enzymes and microorganisms, and it is these supports that were chosen as component *4* to be included inside the composite biocatalyst. However, the biocatalyst granules containing Sibunit disintegrated rapidly on contact with the aqueous reaction medium. For this reason, the following carbon materials were examined systematically: CFC synthesized on supported Ni and Cu catalysts [17] and carbon nanotubes (CNTs) synthesized on a $Mg_{0.99}(Co_{¾}Mo_{¼})_{0.11}O$ catalyst (8–9 wt %) [19, 20]. CFC granules form as a result of the interlacing and densification of carbon nanofibers (CNFs) [17]. The BET surface area (S_{BET}) of CFC was 162 m^2/g, ant its mean pore diameter was 10–50 nm. Before use, CFC granules were thoroughly ground into a fine powder. CNTs were a fine powder with $S_{BET} = 105$ m^2/g.

The specific surface area of the supports was determined by thermal nitrogen desorption using a Sorbi-M instrument (Meta, Russia). Pore size distribution in the supports was studied by mercury porosimetry on an AutoPore 9200 porosimeter (Micromeritics, United States). Glucose isomerase partially purified from *A. nicotianae* and rec-*E. coli* and the recombinant enzyme His_6-GI obtained by Rozanov et al. [8] were adsorbed from buffer solutions (0.02 M phosphate, pH 7.8 and pH 7.0) onto inorganic supports. This was done by occasionally stirring the enzymatic protein solution with a support (10 : 1 by weight) over 1 day at 20–22°C. The adsorbents were chemically different mesoporous supports: θ-Al_2O_3 ($S_{BET} = 55$ m^2/g), SiO_2 (Silokhrom S-1.5 brand, $S_{BET} = 66$ m^2/g), Sibunit ($S_{BET} = 550$ m^2/g) [16], and carbon-containing aluminum oxide (SUMS-1) obtained by catalytic pyrolysis of divinyl on γ-Al_2O_3 (20% pyrocarbon, $S_{BET} = 200$–220 m^2/g) [20].

The GI activity of biocatalysts was measured at 70°C (50°C for His_6-GI) in a 0.02 M phosphate buffer solution (pH 7.0, 1 mM Mg^{2+}, 1 mM Co^{2+}). In activity measurement for the heterogeneous biocatalysts containing insoluble Co_xO_y, Co^{2+} ions were not added to the reaction medium. The substrates were 2–3 M fructose solutions. The reaction rate 1 μmol/min was accepted to be the enzymatic activity unit (U). Activity was expressed in terms of the number of activity units per gram of dry cells and per gram of dry biocatalyst for suspended and immobilized cells, respectively. The glucose concentration resulting from fructose isomerization in the reaction medium was determined by a glucose-specific method using glucose oxidase [22]. The GI activity of suspended cells and the specific activity of the partially purified enzyme at 70°C were 2000–4000 U/(g dry cells) and 8000–12000 U/(g protein), respectively.

The activity of heterogeneous biocatalysts (**A**) was measured using a closed-loop circulation setup consisting of a differential gradientless reactor as a glass column with a thin biocatalyst bed ($g = 0.1$–0.5 g), a magnetically stirred mixer, a thermostat maintaining the preset temperature (70°C) of the reaction mixture in the mixer and in the biocatalyst bed, and a peristaltic pump for circulation of the substrate solution through the biocatalyst at a rate of 1.0–1.5 ml/min. The duration of one reaction cycle was 2–8 h. Thereafter, the reaction medium was removed and the biocatalyst was washed with distilled water and with the buffer solution (pH 7.0). For freshly prepared biocatalyst, we determined the reaction rate in a reaction mixture aliquot sampled from the mixer after 1-h-long circulation and held for another 1 h at 70°C in the absence of a biocatalyst, thus completing a 2-h-long reaction cycle (w_{medium}). The overall reaction rate (w_Σ) was the sum of w_{medium} and the reaction rate in the presence of the biocatalyst (w_{cat}); that is, $w_{cat} = w_\Sigma - w_{medium}$. The initial activity of biocatalysts (A_0) was estimated from the overall reaction rate as $A_0 = w_\Sigma/g$. After biocatalyst conditioning, when $w_{medium} \approx 0$, we calculated the steady-state activity as $A_{st} = w_{cat}/g$. Obviously, the steady-state activity is the most significant and practically important characteristic of a biocatalyst, for it characterizes its operating stability and total productivity.

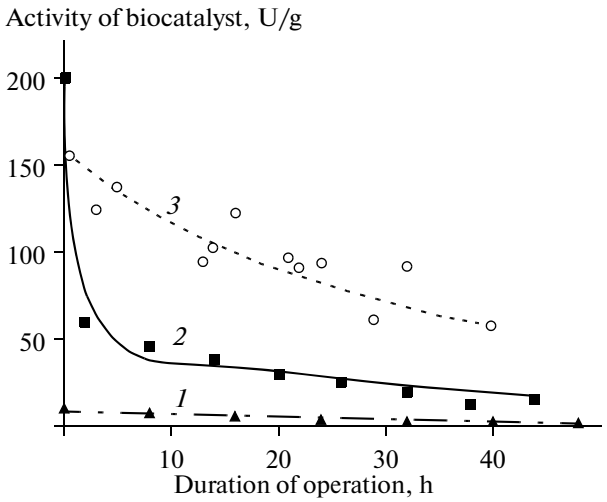

Fig. 2. Activity of heterogeneous biocatalysts in fructose isomerization as a function of working hours: (*1*) *A. nicotianae* inside SiO_2 xerogel at 70°C, (*2*) rec-*E. coli* inside SiO_2 xerogel at 70°C, and (*3*) Sweetzyme T at 60°C.

The continuous isomerization of monosaccharides (glucose, fructose) was carried out in a plug-flow reactor with a fixed bed prepared from 1–4 mm granules of a biocatalyst and an inert filler (glass balls 2 mm in diameter) taken in a 1 : 1 volume ratio. The filler substantially reduced the flow resistance of the bed. The reactor was placed in a thermostat maintained at 62 ± 2°C, and a 3 M fructose solution was pumped through the bed (10 cm^3) at a rate of 0.02 ml/min. At intervals (one time a day), the reaction mixture was sampled at the reactor outlet and was analyzed for glucose, and the substrate (fructose) conversion was calculated.

The operating stability of biocatalyst was estimated for fructose isomerization in both batch and continuous modes and was characterized by the half-inactivation time ($t_{1/2}$). It was also demonstrated that, as the biocatalysts are stored in the buffer solution at 20–22°C over the time interval between reaction cycles of the batch process (18–20 h), they practically do not lose their activity.

RESULTS AND DISCUSSION

As freshly prepared biocatalysts are conditioned in the reaction mixture, the reaction rate w_Σ decreases by a factor 5–10 in the initial 1–2 h (Fig. 2, curve *2*). In some cases, for example, at a high microbial biomass content of the biocatalyst, $w_\Sigma \approx w_{medium}$, indicating that the enzymatic protein is leaked away from the biocatalysts. Thereafter, the biocatalysts operate rather stably for a long time with a steady-state activity A_{st}. A comparison of the rate curves for the biocatalyst based on the *A. nicotianae* strain (Fig. 2, curve *1*) and recombinant *E. coli* strain (Fig. 2, curve *2*) and the rate curve for Sweetzyme T biocatalyst (Fig. 2, curve *3*) shows how greatly and rapidly the initial reaction rate falls in the case of the biocatalyst based on rec-*E. coli*. Mathematical processing of the rate curves presented in Fig. 2 demonstrated that curves *1* and *3* can be fitted well to an exponential function characteristic of a first-order reaction with an inactivation constant of $k_{in} = 3.8 \times 10^{-2}$ h^{-1} (70°C) and 2.7×10^{-2} h^{-1} (60°C). By contrast, two quite different portion can be distinguished in curve *2*, specifically, the initial portion, in which the reaction rate decreases rapidly, and the flat portion, in which k_{in} is 3.5×10^{-2} h^{-1} (70°C) and almost coincides with the k_{in} value for the biocatalyst based on the gene donor strain of *A. nicotianae*. This coincidence and the large value of w_{medium} during conditioning suggest that the main cause of the decrease in the activity of the biocatalysts based on the *E. coli* strain is the escape of the enzymatic protein from the cells, which are destroyed during the biocatalyst preparation procedure (drying, pressing) and, then, the leakage of the protein into the silica matrix and reaction medium. The fact that the reaction medium remains transparent during the operation of the freshly prepared biocatalyst is further evidence that proteins rather then cells come out of the silicate matrix. If whole and/or destroyed bacterial cells were leaked out of the biocatalyst in an amount capable of ensuring the observed w_{medium} value, then, according to our estimates, the turbidity of the reaction medium would be noticeable, with an optical density of 0.4 at 590 nm (in 1 cm-cuvette).

In order to enhance the steady-state activity of the GI biocatalysts, we used various methods and experimental techniques, such as heat fixation of intracellular GI at elevated temperatures, cross-linking with GA, treatment of the dry biocatalyst with silane, re-inclusion of the prepared biocatalyst inside SiO_2 xerogel, and preparation of composites by including a carbon-containing component *4* as an adsorbent inside the silica matrix.

The GI produced by natural microorganisms is localized near the cell wall and is bound to its murein framework. One way of hampering the escape of the intracellular enzymatic protein outside is by fixing it inside the cells, as was demonstrated for an *A. nicotianae* strain [23]. This can be done either by elevating the temperature (heat fixation) or by treating the strain with a solution of a salt (KCl, NaCl, LiCl, or $MgCl_2$) or organic (citric) acid. For example, heat treatment of *A. nicotianae* biomass at 60 and 70°C for 47 and 15 min, respectively, raises the GI activity of the cells by a factor of 1.5–2.6 relative to that of the intact (non-heated) cells [23]. Similar experiments were carried out in this study for rec-*E. coli*.

In order to optimize the temperature and duration of GI heat fixation in rec-*E. coli* cells, we initially investigated the thermal inactivation processes in cell suspensions and estimated the thermal inactivation constants (k_{in}^T). The k_{in}^T values for the cells heat-treated in the buffer solution at 75, 80, and 85°C are

Table 1. Properties of the biocatalysts based on intact and heat-treated rec-*E. coli* cells*

GI heat fixation conditions (duration of heat treatment of biomass in a buffer solution at pH 7.0)	A_0 (70°C) for the freshly prepared biocatalyst, U/g	A_{st}, U/g	$t_{1/2}$ (70°C), h
Control (no heating)	554	117	9
70°C (2 h)	398	44	8
75°C (2 h)	556	48	8
75°C (2 h) + 85°C (10 min)	544	19	6

* Biocatalyst composition: biomass : SiO_2 : Co_xO_y = 45 : 50 : 5 (wt %).

3.2×10^{-3}, 6.3×10^{-3}, and 1.8×10^{-2} min^{-1}, respectively. Based on experimental data on the thermal stability of GI, we chose heat fixation conditions (Table 1). It was found that, irrespective of GI heat fixation conditions, the biocatalysts show similar initial activities, but they are characterized by different values of steady-state activity, which decreases with increasing heat fixation temperature (Table 1). After 14-h-long operation at 70°C, the catalytic activity was 21% (without heat fixation) and 3–11% (with heat fixation) of the initial activity A_0. Thus, the heat fixation of intracellular GI does not improve the properties (activity and stability) of the biocatalysts prepared using rec-*E. coli*.

Cross-linking with GA was carried out both for wet biocatalysts mixture (*1* + *2* + *3* + (*4*)) prepared ("wet" cross-linking), as was described in our earlier work [11], and for the already prepared biocatalyst by treating its dry granules by a GA solution ("dry" cross-linking). It was demonstrated earlier [11] that the optimum way of preparing biocatalysts using wet cross linking is as follows: a GA solution is added to the two-component uniform mixture (*2* + *3*) so that the dialdehyde content is 20–40 mg per gram of dry cells, and then microbial biomass (*1*) is added. With this biocatalyst preparation procedure, A_0 and A_{st} are approximately equal and $A_{st} \approx$ 100–110 U/g. As the GA concentration is increased, the activity of the biocatalysts decreases by a factor of 10–50, for example, from 110 U/g at a GA content of 40 mg/g to 12 U/g at a GA content of 240 mg/g. Therefore, GA inactivates GI. This is likely due to the fact that GA interacts not only with the NH_2 groups of the amino acid residues of the protein molecule, but also with the NH_2 groups of the amino acids involved in active site of enzyme and responsible for catalytic action. Two asparagine residues—Asp104 and Asp339—are indeed amino acids of the active site of the glucose isomerase [24, 25]. It was demonstrated that, in the case of dry cross-linking, the initial activity decreases from 500–700 to 130–150 U/g, while the A_s value is practically independent of the GA concentration in the 0.1–1% range and is, on the average, 150 U/g (Fig. 3). The dry cross-linking of the biocatalyst with GA allowed the stead-state activity to be increased by a factor of ~1.5 relative to the activity of the non-cross-linked biocatalyst (Fig. 3) and by a factor of ~2 relative to the activity of the wet-cross-linked biocatalyst. For example, for the same amount of GA per gram of dry cells (140–160 (mg GA)/g), the activity of the biocatalyst was 150 and 80 U/g, respectively. Evidently, dry cross linking is preferable to wet cross linking, since in the former case the concentration of the inactivating agent and its distribution in the biocatalyst volume are controlled more accurately and this ensures good data reproducibility. It is possible that, if GA did not exert a pronounced inactivating effect on GI, the results of dry cross linking would be more significant.

The biocatalysts were treated with γ-APTES in order to generate additional sites for binding the GI molecules through their interaction with grafted NH_2 groups. As a result of the treatment of the biocatalyst with γ-APTES, the initial activity decreased from 530 to 170 U/g and the steady-state activity was ~100 U/g. Additional cross linking with 1% GA further reduced the activity from 170 to 50 U/g. Thus, the treatment of

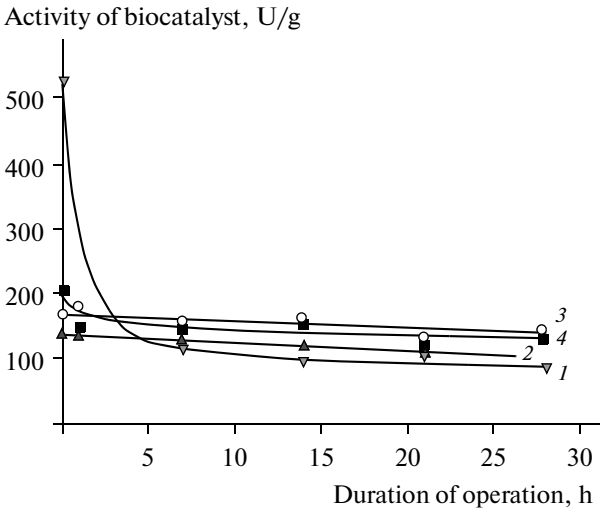

Fig. 3. Activity of biocatalysts prepared by dry cross-linking with GA under various conditions as a function of working hours: (*1*) no cross-linking, (*2*) cross-linking with a 1% GA solution, (*3*) cross-linking with a 0.2% GA solution, and (*4*) cross-linking with a 0.1% GA solution.

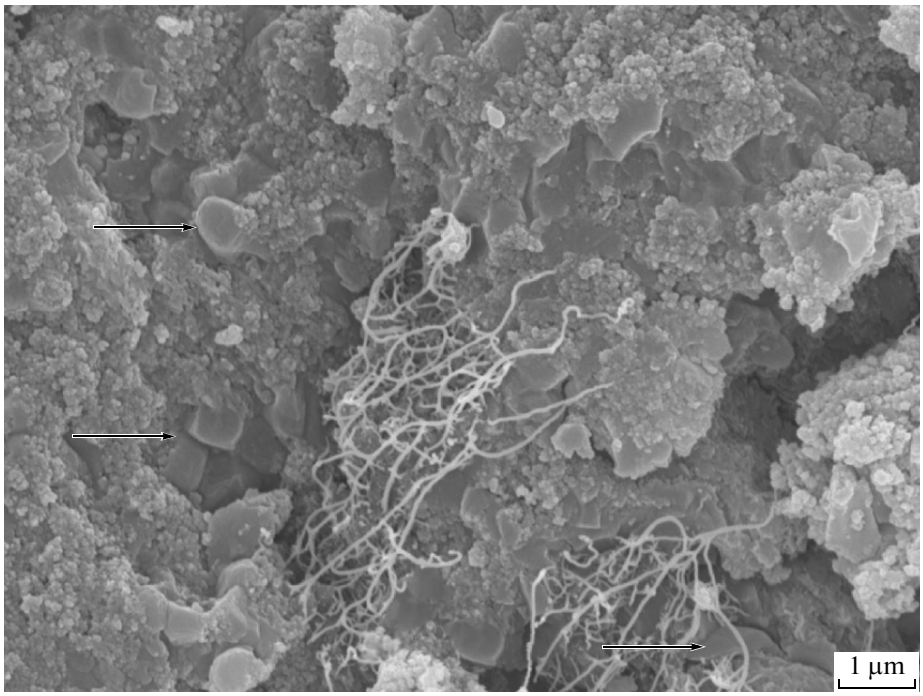

Fig. 4. Electron micrograph of a cleavage of a carbon–silica composite biocatalyst prepared by inclusion of rec-*E. coli* cells (shown with arrows) into SiO_2 xerogel containing 5 wt % CNTs.

the biocatalysts with chemical reagents (GA or γ-APTES) markedly decreases their GI activity.

In the case of double inclusion of microbial biomass into SiO_2 xerogel, the initial isomerization rate is proportionally lower (by a factor of 5) and the reaction rate decrease in the same way as is shown by curve *2* in Fig. 2. This indicates that, in this case, the enzymatic protein passes from the biocatalyst into the reaction medium as well. In order to keep the enzyme in the biocatalyst, we used nanocarbon-containing materials as the adsorbent and component *4*. It was demonstrated in earlier works [12–14] that the CFC formed as a result of the interlacing and densification of nanofibers is an efficient adsorbent for various enzymatically active substances, including enzymes (glucoamylase) and bacterial cells. Determination of the amino acid sequence of the GI molecule of *A. nicotianae* demonstrated that the enzyme contains 73.2% hydrophobic amino acids and 26.8% hydrophilic ones [6, 7]. Therefore, the enzyme molecule possesses pronounced hydrophobic properties, which can make it readily adsorbable on the comparatively hydrophobic surface of the carbon material.

The mechanical strength of biocatalyst granules and their resistance to disintegration in an aqueous reaction medium at pH 7.0 are correlated with the compactibility of component *4*. For example, Sibunit powder could not be pressed into pellets and the biocatalyst granules containing Sibunit as component *4* disintegrated totally in the reaction medium as soon as 2 h after the process was started. At the same time, the inclusion of the readily pelletizable CNFs or CNTs made it possible to prepare mechanically strong, disintegration-resistant biocatalysts capable of operating stably in buffer reaction media at neutral pH. The electron micrographs of cleavages of these composite biocatalysts (Fig. 4) clearly show *E. coli* cells embedded in silicon dioxide xerogel, as well as CNTs.

Comparative studies demonstrated that the inclusion of CNFs and CNTs into the biocatalysts does change the biocatalytic properties of the latter only slightly. A favorable effect was observed for the biocatalysts prepared using CNFs. The A_{st} values of the carbon-containing biocatalysts differed from those of the biocatalysts containing no component *4* by 10–15%, with a systematic deviation toward higher values of the measured parameters. The kinetics of the reaction in the presence of carbon–silicate composite biocatalysts was nearly the same as the kinetics of the reaction in the presence of the carbon-free biocatalysts. The steady-state activity was ~100 U/g. With the CNT-containing biocatalysts, the initial reaction rate was ~2 times lower than the reaction rate in the presence of CNF-containing biocatalysts. This is possible due to the presence of molybdenum in the $Mg_{0.99}(Co_{¼}Mo_{¾})_{0.01}O$ catalyst used in CNT synthesis [19, 20]. In the case of the dry cross-linking of the carbon–silica biocatalysts, the steady-state activity is ~80–90 U/g; that is, component *4* does not protect GI against the inactivating effect of GA.

We investigated why the nanocarbon materials introduced into the silicate matrix exert only a slight positive effect. It was hypothesized that the GI mole-

Table 2. Properties of the biocatalysts prepared by GI adsorption on inorganic supports

Support	S_{BET}, m^2/g	S_{adsd}* m^2/g	GI from *A. nicotianae*			GI from rec-*E. coli*			His_6-GI		
			amount adsorbed, *a*, mg/g	A_0(70°C), U/g	$t_{½}$, h	amount adsorbed, *a*, mg/g	A_0(70°C), U/g	$t_{½}$, h	amount adsorbed, *a*, mg/g	A_0 (70°C), U/g	$t_{½}$, h
θ-Al_2O_3	55	18	5.3	5.3	8	–	–	–	–	–	–
SiO_2	66	60	13.0	16.9	2	27	1.8	<2	–	–	–
SUMS-1	200	7	3.6	<0.1	<1	–	15.8	<2	6.4	<0.1	<2
Sibunit	550	44	8.0	1.6	–	–	–	–	22.2	0.45	7

* Calculated from mercury porosimetry data as the specific surface area of the support minus the surface area of the pores smaller than 20 nm in diameter.

cules are weakly adsorbable on solid supports. The adsorbability of the enzymatic protein was not studied earlier, and it was believed that, for preparing commercial biocatalysts, it is appropriate to use the purified enzyme because it has a higher specific GI activity than in cell suspensions. As was noted above, amino acid sequencing demonstrated that the enzyme from *A. nicotianae* contains 73.2% hydrophobic amino acids and 26.8 hydrophilic ones [7, 8]. Out of the hydrophilic amino acids, 54.7% are negatively charged and 45.3% are positively charged. The isoelectric point of the enzyme molecule lies in the acidic region, so at neutral pH values the molecule bears a small negative charge. There have been direct experimental studies on the adsorptive immobilization of partially purified GI from *A. nicotianae* and rec-*E. coli* and of the recombinant enzyme with six terminal histidine residues (His_6-GI) [8] on chemically different mesoporous inorganic supports, namely, θ-Al_2O_3, SiO_2 (Silokhrom type), and the carbon-containing materials SUMS-1 and Sibunit (Table 2). At pH 7.0, the alumina surface is positively charged and the surfaces of silica gel and oxidized carbon near a slight negative charge. The carbon supports show hydrophobic properties.

An analysis of the textural characteristics of the supports and adsorption data suggests that the key factor in the adsorption immobilization of GI is the surface area accessible to the adsorbate (S_{ads}) rather than the chemical nature of the support. The S_{ads} value can be estimated by mercury porosimetry as the specific surface area minus the surface area of the pores smaller than 20 nm in diameter. The molecule of GI from *A. nicotianae* is a tetramer consisting of four identical subunits each having a mass of 190 kDa [7, 8, 25]. The size of this protein molecule in solution is at least 20 nm. The S_{ads} was estimated under the assumption that only pores over 20 nm in diameter are accessible to this molecule. It was demonstrated that, the larger the S_{ads} value, the larger the amount of protein adsorbed (Table 2). The amount of protein adsorbed (***a***) is largest for Silokhrom ($S_{BET} \approx S_{ads} = 60$ m^2/g), and, under the conditions examined, $a = 13$ and 27 mg/g for the proteins from *A. nicotianae* and rec-*E. coli*, respectively (Table 2). From the data presented in Table 2, we estimated the protein adsorption density expressed in terms of milligrams of protein per square meter of the accessible surface S_{ads}. It was found that, with the errors of the measurement of S_{ads} and protein concentration in solution taken into account, the adsorption density is nearly the same for all of the adsorbents and is ~0.3 mg/m^2. It was calculated that, at this adsorption density, the radius of an adsorbed protein molecule with a mass of 200 kDa is ~18 nm, indicating the formation of a monolayer coverage on the support surface.

The specificity of GI adsorption on the support surfaces was evaluated by measuring the enzymatic activity before and after contact between an enzyme-containing solution and the support. It was found that the specific GI activity after sorption is 1.5–2.5 times higher than A_{sp} in the solution before adsorption. This is unambiguous evidence that impurity proteins accompanying the partially purified enzyme are mainly adsorbed on the Silokhrom and Sibunit surfaces. The θ-Al_2O_3 surface adsorbs GI and accompanying proteins to equal extents: the specific GI activities in the solution before and after adsorption are similar (17 and 16 U/g, respectively).

Upon adsorption, GI loses over 90% of its activity observed in solution, undergoing almost complete inactivation on carbon supports (Table 2). The active site of GI mainly involves hydrophobic amino acids (tryptophan Trp188 and Trp130 and histidine His101) [23]. For adsorption on the carbon surface, which has pronounced hydrophobic properties, the likely orientation of the protein molecule is the one in which its active site is bound to the surface owing to hydrophobic interaction and is thus blocked, becoming inaccessible to the substrate. This prevents the formation of an enzyme–substrate complex and makes impossible biocatalysis.

The activities of the biocatalysts prepared by GI adsorption on inorganic supports differ significantly, depending on the microbial source of the enzyme (Table 2). The biocatalyst prepared by adsorbing GI from *A. nicotianae* onto Silokhrom shows the highest initial activity, $A_0 = 17$ U/g, owing to the fact that the amount of enzyme adsorbed and the specific activity of the enzyme on this support are relatively high (Table 2).

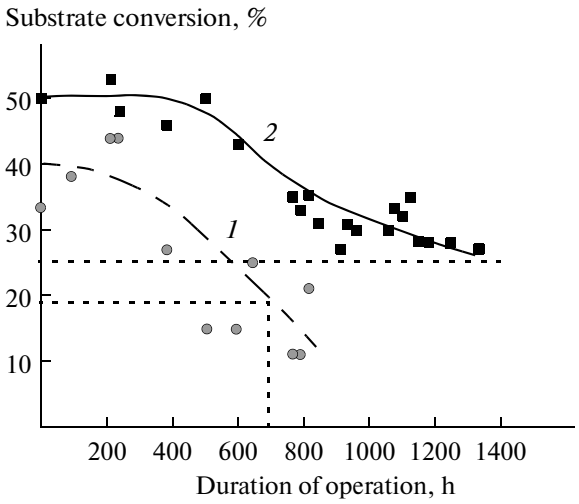

Fig. 5. Substrate conversion in continuous isomerization as a function of duration of operation for biocatalysts based on (*1*) *A. nicotianae* and (*2*) rec-*E. coli*. Process conditions: 62 ± 2°C, 3 M fructose, 1 mM Mg^{2+}, 0.05 M phosphate buffer, pH 7.8 for *A. nicotianae*, pH 7.0 for rec-*E. coli*.

Conversely, for GI from rec-*E. coli*, the biocatalyst prepared by adsorption on Silokhrom shows the lowest initial activity, A_0 = 2 U/g (Table 2), although the amount of enzyme adsorbed is comparatively large (27 mg/g). A similar situation is observed for the biocatalysts prepared using carbon-containing aluminum oxide SUMS-1: GI from *A. nicotianae* undergoes complete inactivation on being adsorbed on this support, while the biocatalyst based on GI from rec-*E. coli* shows a comparatively high initial activity of ~16 U/g (Table 2). The observed distinctions may be due to the presence of proteins impurities and their nature in the GI-active samples studied.

Both the activity and operating stability of the biocatalysts prepared by GI adsorption are extremely low. *A. nicotianae*'s GI adsorbed on Silokhrom or Sibunit is completely inactivated within two 2-h-long operation cycles. The maximum half-inactivation time observed for the biocatalyst prepared by GI adsorption on θ-Al_2O_3 and by His_6-GI adsorption on Sibunit does not exceed 7 h (Table 2). It was established that the main cause of the inactivation of these biocatalysts is enzyme desorption from the support surface. It was observed for Silokhrom that, in the first reaction cycle, w_{medium} is 2 times higher than w_{cat}. Thus, the properties of the protein molecule and the properties of the support surface are correlated neither with the ability of GI to adsorb on this surface nor with the biocatalytic properties of adsorbed GI.

The discovered properties of the enzymatic protein GI, namely, nonspecific and weak adsorption and loss of up to 90–100% of the enzymatic activity upon adsorption is the main reason why the introduction of a carbon material into the biocatalysts does not produce an additional positive effect. The steady-state activity and operating stability of the composite carbon–silica biocatalysts differ from the same characteristics of the biocatalysts containing no carbon component by a factor no grater than 1.2. Note, however, that a similar study on yeast autolysate, another enzymatically active substance adsorbable on carbon materials, has demonstrated that the introduction of carbon into the biocatalyst produces a considerable positive effect, making it possible to raise the steady-state invertase activity several times.

Testing the catalyst prepared using the recombinant producer strain of *E. coli* and a silicate or carbon–silicate matrix in continuous monosaccharide isomerization demonstrated that the half-inactivation time of these biocatalysts is ~1500 h, which is more than 2 times longer than $t_{½}$ for the biocatalysts prepared using the natural *A. nicotianae* strain (Fig. 5). The steady-state activity ensuring the stable and productive operation of the heterogeneous biocatalyst subjected to dry cross-linking with GA is 150–160 U/g at 70°C, which is more than 4 times higher than the activity of the catalysts based on *A. nicotianae* [26]. The total productivity of this biocatalyst is estimated at 1.5–2 (t GFS)/kg.

Undoubtedly, novel composite matrices obtained using nanocarbon materials will find application in various fields of biotechnology, including heterogeneous biocatalysis.

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