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Immobilization of glucoamylase by adsorption on carbon supports and its application for heterogeneous hydrolysis of dextrin

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Abstract—Glucoamylase (GA) was immobilized by adsorption on carbon support: on Sibunit, on bulk catalytic filamentous carbon (bulk CFC) and on activated carbon (AC). This was used to prepare heterogeneous biocatalysts for the hydrolysis of starch dextrin. The effect of the texture characteristics and chemical properties of the support surface on the enhancement of the thermal stability of the immobilized enzyme was studied, and the rates of the biocatalyst's thermal inactivation at 65–80 °C were determined. The thermal stability of glucoamylase immobilized on different carbon supports was found to increase by 2–3 orders of magnitude in comparison with the soluble enzyme, and decrease in the following order: GA on Sibunit > GA on bulk CFC > GA on AC. The presence of the substrate (dextrin) was found to have a significant stabilizing effect. The thermal stability of the immobilized enzyme was found to increase linearly when the concentration of dextrin was increased from 10 wt/vol % to 50 wt/vol %. The total stabilization effect for glucoamylase immobilized on Sibunit in concentrated dextrin solutions was about 10^5 in comparison with the enzyme in a buffer solution. The developed biocatalyst, 'Glucoamylase on Sibunit' was found to have high operational stability during the continuous hydrolysis of 30–35 wt/vol % dextrin at 60 °C, its inactivation half-time ($t_{1/2}$) exceeding 350 h. To improve the starch saccharification productivity, an immersed vortex reactor (IVR) was designed and tested in the heterogeneous process with the biocatalyst 'Glucoamylase on Sibunit'. The dextrin hydrolysis rate, as well as the process productivity in the vortex reactor, was found to increase by a factor of 1.2–1.5 in comparison with the packed-bed reactor.

Keywords: Glucoamylase; Carbon supports; Adsorption; Heterogeneous biocatalyst; Thermal stability; Dextrin hydrolysis

1. Introduction

A heterogeneous biocatalyst prepared by the immobilization of glucoamylase (1,4-α-D-glucan-glucohydrolase, EC 3.2.1.3) and designed for one of the key stages of starch conversion—saccharification (hydrolysis of dextrin)—can become the basis for the development of a new modern technology for production of sweeteners (treacle with variable hydrocarbon composition and glucose syrups) from renewable vegetable feedstock. For successful commercialization of the heterogeneous process of starch saccharification, a biocatalyst must convert 45% of the substrate in 15–20 min with an inactivation half-time ($t_{1/2}$) 30–120 days, which corresponds to a 3–12 month operation of the reactor without reloading.[1] A high value of $t_{1/2}$ ($\geqslant$50–100 days) is essential to reduce the biocatalyst cost and to increase biocatalyst productivity up to a recommended value from 100 to 10,000 kg of substrate converted per kg of biocatalyst.[2] The most important characteristic for the industrial application of a biocatalyst is its high thermal stability at a pasteurization temperature of 60 °C or higher. In the 1970s Corning Glass Co. carried out pilot plant tests of a packed-bed reactor with a glucose productivity of 450 kg per day. The reactor was filled with a biocatalyst prepared by covalent binding of glucoamylase onto the surface of macroporous silica.[3] Inactivation half-times of this biocatalyst at 60 °C and 70 °C were 150 h and 7.5 h, respectively.[3] According to the opinion of the specialists working on this project, it is

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the low thermal stability of the immobilized glucoamylase that was the main reason why this process was not commercialized. It is important to mention that no biocatalyst based on immobilized glucoamylase with satisfactory thermal stability at $\geqslant 60$ °C has been developed to date. The best result obtained so far is the inactivation half-time 300 h at 50 °C obtained for a biocatalyst prepared by immobilization of GA on polystyrene.[4]

Another factor limiting the commercialization of heterogeneous biocatalysis is the relatively low productivity of packed-bed reactors, particularly in processes controlled by the substrate diffusion to the immobilized enzyme. The results of the studies on the development of new types of bioreactors may be used as the basis for the modernization of the existing technology or development of new competitive ones. The most important problem that has to be solved by the new reactor design is the significant decrease of diffusion limitation by providing intensive forced mass transfer of the substrate to the biocatalyst. This mass transfer can be intensified, for example, by the creation of a vortex movement in the liquid. The idea to use Taylor–Poiseulle vortex flow for carrying out heterogeneous biocatalytic processes with immobilized enzymes and the theoretical background for the construction of such bioreactors was suggested by Iosilevskii.[5] Later this idea was implemented in a vortex flow reactor that consisted of two cylinders, the inner cylinder being immersed into the substrate solution and rotated in it.[6–8]

Earlier we have studied the biocatalytic properties of glucoamylase immobilized on macrostructured and granular carbon-containing alumina–silica and alumina supports differing by the morphology of the carbon layer synthesized on the surface.[9,10] We have shown that the thermal stability of glucoamylase adsorbed on a honeycomb monolith coated by carbon nanofibers increases by a factor of 20 in comparison with the soluble enzyme; the inactivation half-time of this biocatalyst at 65 °C was 9.5 h.[9] Earlier we had studied the biocatalytic properties of glucoamylase immobilized on granular pure carbon supports, including Sibunit, graphite, and sapropel.[10,11] Mesoporous Sibunit was found to be the most efficient adsorbent for glucoamylase.[10] In the present study, we have continued a comparative investigation of granular pure carbon supports for the adsorptive immobilization of enzymes.

The present study was devoted to the investigation of the properties (activity and stability) of biocatalysts prepared by glucoamylase adsorptive immobilization on granular pure carbon supports: Sibunit, bulk catalytic filamentous carbon (bulk CFC), as well as activated carbon (AC). The constants of thermal inactivation of soluble and immobilized glucoamylase were determined and compared. The effect of the substrate (dextrin) on the thermal stability of immobilized glucoamylase was studied. The operational stability of the developed biocatalyst 'Glucoamylase on Sibunit' under model technological conditions of starch saccharification was determined. An immersed vortex reactor (IVR) of a new type was designed and tested in starch saccharification by the biocatalyst 'Glucoamylase on Sibunit'. A lab-scale scheme for starch bioconversion was developed and tested.

2. Experimental

2.1. Materials

2.1.1. Enzyme and substrate. Glucoamylase from a commercial sample of Glucoawamorin® ('Sibbiopharm', Novosibirsk, Russia) was used for adsorptive immobilization on carbon supports. The specific glucoamylase activity at 50 °C and pH 4.6 was equal to ~200 U/mg of protein.

The dextrin used as a substrate for soluble and immobilized glucoamylase was prepared as follows: Dry potato, corn, or wheat starch was mixed with a dry enzyme sample of α-amylase, Amylosubtilin® ('Sibbiopharm', Novosibirsk, Russia), and carefully homogenized. The mixture thus obtained was suspended in distilled water, heated under intensive stirring to 85 °C, and maintained at this temperature for 15–20 min. At the end of this time the mixture was boiled for 5 min to inactivate the α-amylase, then cooled to room temperature, and filtered. The concentration of dry-weight substances in the substrate solutions (wt/vol %) was measured using an RL3 refractometer ('PZO Warszawa', Poland). The molecular weight of the prepared dextrin was estimated at 3–5 kDa. The dextrose equivalent (DE) of dextrin substrate was determined to be equal to ca. 10.

2.1.2. Supports. Sibunit® and bulk CFC were developed at the Boreskov Institute of Catalysis as supports and adsorbents with desired regulated properties.[12–14] These supports were earlier studied for the immobilization of some biologically active substances (amino acids, proteins, and non-growing bacterial cells).[15,16] Activated carbon (AC) is a commercial product that is used as an adsorbent in the food industry for the decolorization of sugar syrups and for drinking water purification. The physical characteristics of the supports in the study are shown in Table 1.

2.2. Methods

2.2.1. Method of enzyme immobilization. The immobilization of glucoamylase on carbon supports was carried out by adsorption under static conditions at 18–22 °C as described earlier.[10,11]

Table 1. Texture characteristics of the carbon supports

Support	$S_{SP\ BET}$, m^2/g[a]	S_{SP}, m^2/g (without micropores)[b]	V_{Σ} pore (without micropores) mL/g[b]	Predominant pore diameter, nm[b]	Accessible surface area S_{ACC}, m^2/g[c]	Accessible pore volume, mL/g[c]
Sibunit	555	95	0.86	18	92	0.85
Bulk CFC	162	67	0.38	11	42	0.34
AC	1296	49	0.53	4	9	0.47

[a] Determined by BET method using thermal desorption of argon.
[b] Determined by mercury porosimetry.
[c] Calculated from pore size distributions data for pores larger than 10 nm.

2.2.2. Characterization of supports. The specific surface areas of the carbon supports were measured by the thermal desorption of argon using a Sorbi-M instrument ('Meta', Russia). The pore size distributions were determined by mercury porosimetry using an Auto-Pore 9200 instrument ('Micromeritics', USA). The texture characteristics of the carbon supports are reported in Table 1.

The surface acid–base properties of the adsorbents used were studied by potentiometric titration at 20–22 °C using acetic (pK_a 4.75) and hydrochloric acids, and ammonium (pK_b 4.75) and sodium hydroxides as indicators for measuring basicity and acidity, respectively. Support samples with surface areas of 50–100 m^2 were immersed into the indicator solutions and maintained under stirring for 30 min to obtain stationary pH values. The calculations of concentrations of the acid/base surface sites expressed in nmol/m^2 were performed in the monolayer coverage region corresponding to the plateau on the Langmuir isotherms obtained.

The hydrophobic properties were evaluated by naphthalene adsorption from its saturated solution at 20–22 °C. Support samples with 1 m^2 surface area were degassed using a water-jet pump to improve their wettability, then put in contact with 0.09 mM naphthalene solution for 60 min. The naphthalene concentrations were measured spectrophotometrically at 220 nm and were calculated using extinction coefficient 1.32×10^5 L cm^{-1} mol^{-1}. The amounts of adsorbed naphthalene were determined from the decrease of its concentration and expressed in nmol/m^2.

2.2.3. Measurements of glucoamylase activity. Standard conditions commonly used for the measurement of the activity of soluble and immobilized glucoamylase were the following: 50 °C, 0.05 M acetate buffer pH 4.6, and 10 w/vol % solution of potato dextrin as substrate. The amount of glucose (in μmol) generated for 1 min was used as the Unit of enzyme activity (U). The specific enzyme activities were calculated as the amount of U per mg of the protein (U/mg) and as the amount of U per g of the dry-weight biocatalyst (U/g) for soluble and immobilized GA, respectively. The dextrin hydrolysis by immobilized GA was carried out using reactors of the following types: (1) A differential gradientless reactor was used to determine the thermal inactivation kinetic constants. Due to a thin bed, the degree of substrate conversion during one pass through the biocatalyst did not exceed 0.1%. As a result there were no gradients of the product and substrate concentrations along the biocatalyst bed. (2) A packed-bed reactor was used to determine the stability of the biocatalysts. The height of the biocatalyst bed was varied from 0.5 to 5.7 cm. Reactors (1) and (2) were a temperature-controlled glass column with a length of 10-cm and a diameter of 1-cm filled with a biocatalyst. The substrate solution was circulated through the biocatalyst bed using a peristaltic pump with a flow rate that was varied from 3 to 550 mL/min. (3) An immersed vortex reactor (IVR) was used to test this new reactor type in dextrin hydrolysis. Its construction and principle of operation are described in an article from this laboratory.[17] The rotation rate (ω) of the reactor body filled with a biocatalyst was varied from 50 rpm to 1500 rpm. A biocatalyst with 2–4 mm granules was placed in the lower plate of the body, and the upper plate was screwed on its top. For finely dispersed supports a toroidal Capron filter was prepared, filled with the biocatalysts, and placed inside the reactor body. Then, the body was immersed into a flask filled with 300–500 mL of the substrate solution. Its temperature was controlled at 50 ± 5 °C. Specially designed baffles were used to prevent the formation of a cone crater in the twisted liquid. The substrate solution was sucked trough the bottom outlet in the reactor body and was circulated through the biocatalyst bed multiple times for 1 h. The flow rate of the substrate solution through the biocatalyst bed inside reactor body increased proportional to $\omega^{1/2}$. The reactor design has been protected by a Russian patent.[18]

2.2.4. Measurements of stability of immobilized enzyme. The thermal stability of soluble glucoamylase and its inactivation constant (k_{in}^{E}) were determined by Glucoawamorin® (2.0 mg of protein/mL) heating at 60–80 °C in a buffer solution of pH 4.6. Aliquots were taken every 15 min and immediately cooled down in an ice bath. Then, the residual glucoamylase activity was measured under the standard conditions at 50 °C as described above.

The thermal stability of immobilized glucoamylase and its inactivation constants in a buffer without substrate (k_{in}^{E}) and in a buffer with substrate (k_{in}^{ES}) were determined as follows: A biocatalyst sample (0.1 g) was heated at 65–80 °C directly in reactor (1) described above for 15–240 min in a circulated buffer solution of pH 4.6 without a substrate and in the presence of the dextrin in the concentration varied from 1 to 53 wt/vol %. Then, the biocatalyst was washed with the cool buffer solution for 3–5 min, and its residual activity was just measured under the standard conditions at 50 °C as described above.

The operational stability of the biocatalyst prepared by the adsorptive immobilization of Glucoawamorin® on Sibunit (named 'Glucoamylase on Sibunit') was determined in a packed-bed reactor under conditions simulating the technological regime of the starch saccharification process. A 32 wt/vol % solution of dextrin was flushed through the biocatalyst bed at 60 °C for the duration of an 8-h reaction cycle. The ratio of the biocatalyst weight to the substrate solution circulated was 1:150. For each cycle the residual glucoamylase activity was measured under the same conditions for the first hour of reactor operation.

3. Results and discussion

3.1. Characteristics of carbon supports

The carbon supports in the study varied in surface morphology (Fig. 1). As is seen from Figure 1a, the surface of Sibunit was formed by round coke deposits of pyrolytic carbon. As is seen from Figure 1b, the surface of bulk CFC was formed by the chaotic interlacing of carbon nanofibers of 50–100 nm diameter and $\geqslant$1 μm length. The resulting surface was very rough. As is seen from Figure 1c, the surface of AC was formed by graphite-like carbon, which gave a smoother surface.

The analysis of the pore size distributions and specific surface areas of the carbon supports showed that mesopores with a size of 20–100 nm, which are suitable for the immobilization of enzyme molecules with a crystallographic size of almost 10 nm, predominated in the pore structure of Sibunit and bulk CFC (Fig. 2a and b). Meanwhile, activated carbon has a bimodal pore structure with the prevalence of macro- and micropores (Fig. 2c). The accessible surface areas (S_{ACC}) of the carbon supports were estimated using the pore size distribution data and assuming that all pores larger than 10 nm are accessible for enzyme adsorption. The calculations gave us the following order of the supports arranged in decreasing accessible surface area: Sibunit (92 m^2/g) > CFC (42 m^2/g) > AC (9 m^2/g) (Table 1).

The carbon supports that were studied possessed pronounced basic properties, and oxygen-containing C=O groups (including lactone) and phenolic hydroxyl groups were present on their surface.[19,20] The total concentration of basic sites on the surface was (1–2) × 10^{-7} mol/m^2, and the fraction of strong basic sites did not exceed 30% (Table 2). No acid groups were observed on the surface of Sibunit and bulk CFC, whereas their concentration on AC was ~20% of the total concentration of the acidic and basic sites (Table 2). The hydrophobic properties of the supports were similar (Table 2). The basicity of the studied supports decreased in the following order: bulk CFC (209 nmol/m^2) > Sibunit (143 nmol/m^2) > AC (72 nmol/m^2) (Table 2).

3.2. Properties of glucoamylase immobilized on the carbon supports

Comparison of the properties of glucoamylase immobilized on different carbon supports showed that the enzyme adsorption on the support enhanced proportionally to the accessible surface area (Table 3), so that a 2-fold increase of the accessible surface area of the support leads to a 2–3-fold increase of the enzyme adsorption. Then, knowing the accessible surface area of the supports (Table 1), as well as the adsorption values and the molecular weight of glucoamylase (100 kDa), it was possible to calculate the apparent size of an adsorbed enzyme molecule. Table 3 shows that when the amount of adsorbed enzyme is increased, the apparent size of the adsorbed enzyme molecules is decreased, that is, the adsorption layer becomes denser. The calculations also showed that the densest layer of enzyme molecules was formed on the surface of

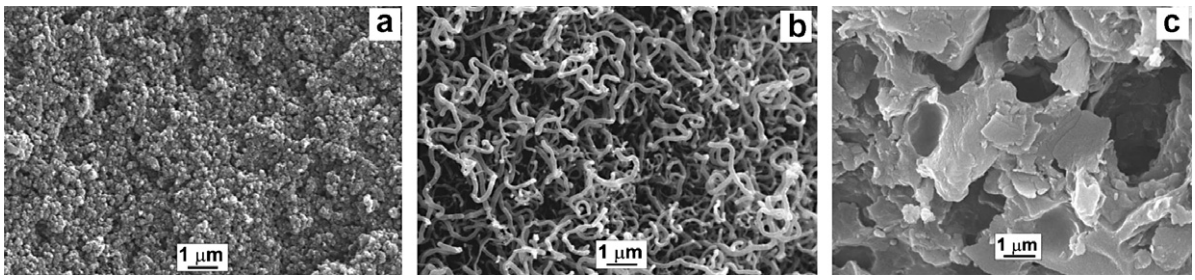

Figure 1. SEM images of the surface of studied carbon supports: (a) Sibunit; (b) bulk CFC; (c) activated carbon.

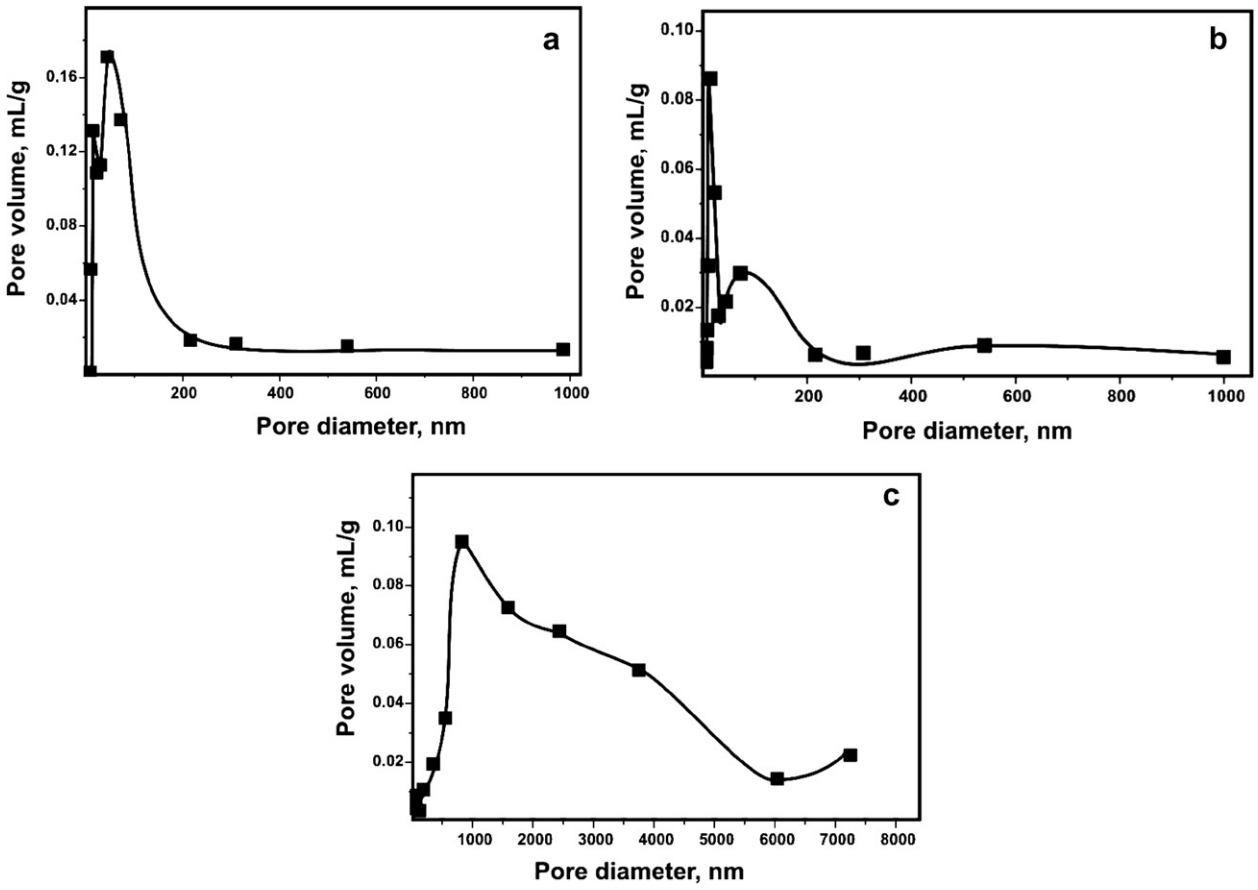

Figure 2. Pore size distribution diagrams of the carbon supports: (a) Sibunit; (b) bulk CFC; (c) activated carbon.

Table 2. Acid–base and hydrophobic properties of the carbon supports

Support	Acidity[a], nmol/m^2		Basicity[a], nmol/m^2		Hydrophobicity, nmol/m^2
	Indicator **1**	Indicator **2**	Indicator **3**	Indicator **4**	
Sibunit	0	0	41	143	184
CFC	0	0	74	209	161
AC	0	21	12	72	152

[a] Indicators: **1**, ammonium hydroxide, pK_b 4.75; **2**, sodium hydroxide; **3**, acetic acid, pK_a 4.75; **4**, hydrochloric acid.

Table 3. Adsorption of glucoamylase on carbon supports and activity of immobilized enzyme

Support (granule size 0.2–1 mm)	Adsorption, mg/g	Diameter of adsorbed enzyme molecule, nm (calculation)	Specific activity, U/mg of protein	Biocatalyst activity, U/g of dry biocatalyst
Sibunit	10.0	39	50.0	500
	12.9	34	32.2	415
	24.1	25	21.9	530
Bulk CFC	4.3	41	90.7	393
	8.2	29	32.7	268
AC	4.6	18	18.3	84

activated carbon. In this case the surface area occupied by one molecule of adsorbed enzyme was close to the size of the hydrated enzyme molecule (~20 nm). Meanwhile, the specific activity of the enzyme immobilized on AC support was lower than on the other studied supports (Table 3). The surface areas occupied by one molecule of enzyme adsorbed on Sibunit and bulk CFC were almost the same (Table 3). However, the enzymatic

activity ran down when the enzyme adsorption increased, and as a result, the adsorption layer became denser (Table 3). Table 3 also shows that the looser structure of the layer formed by the adsorbed protein molecules provides the higher specific activity of the immobilized enzyme. These results seem to indicate that the enzyme active sites are deformed and/or partially blocked in a dense adsorption layer.

Thermal inactivation of glucoamylase adsorbed on the carbon supports has been compared with that of the soluble enzyme. For the latter, the thermal inactivation constant (k_{in}^{E}) and inactivation half-time ($t_{1/2}$) at 65 °C were found to equal 3.3×10^{-2} min^{-1} and 21 min, respectively. At temperatures $\geqslant$70 °C soluble glucoamylase was not stable and quickly became inactivated. Meanwhile, at 65 °C the value of k_{in}^{E} for the immobilized glucoamylase (Table 4) was 2–10 times higher than for the soluble enzyme.

Comparison of inactivation constants of immobilized glucoamylase showed that the enzyme adsorbed on mesoporous Sibunit has the highest stability (Table 4). Glucoamylase adsorbed on Sibunit preserved high enzymatic activity even at 80 °C. At this temperature when it was immobilized on other carbon supports, the enzyme was inactivated relatively fast (Table 4). The constants for thermal inactivation at 75 °C for immobilized glucoamylase increased in the following order: GA on Sibunit (1.9×10^{-2} min^{-1}) > GA on bulk CFC (6.5×10^{-2} min^{-1}) > GA on AC (1.4×10^{-1} min^{-1}). This order was analogous to and matches the ones observed for the decrease of accessible surface area of the support and the amount of mesopores in the pore structure (Table 2). Thus, the above data proved that the pore structure of the carbon supports studied, rather than their hydrophobic–hydrophilic properties, plays the key role in improving the stability of the immobilized glucoamylase. Mesopores matching the size of hydrated enzyme molecules and providing multipoint enzyme binding with the surface of the support are required for the preparation of highly stable biocatalysts.

Thus, these results suggest that mesoporous Sibunit is the most efficient support for the preparation of a highly stable heterogeneous biocatalyst for starch saccharification. All subsequent studies were carried out using biocatalysts prepared by the adsorptive immobilization of Glucoawamorin® on Sibunit that has been named 'Glucoamylase on Sibunit'.

3.3. Properties of glucoamylase immobilized on Sibunit

Note that the values of observed activity of the prepared heterogeneous biocatalysts and specific activity of the immobilized enzymes reported in Table 3 were all measured in the kinetic region for the dextrin hydrolysis reaction. To secure it, we selected the conditions for overcoming diffusion control of the reactions in different types of reactors. For the packed-bed reactor the flow rate of substrate solution through the biocatalyst bed was selected to make sure that the reaction rate did not depend on the flow rate; therefore, there were no external diffusion limitations. As is seen from Figure 3, the process of mass transfer of substrate (dextrin) to immobilized enzyme (glucoamylase) did not influence on the hydrolysis rate at a flow rate more than 30 mL/min. It was found that the greater the height of the bed in the reactor, the greater was the hydrodynamic resistance, and the greater was the flow rate to overcome diffusion restrictions (Fig. 3). All subsequent studies were carried out at 20–30 mL/min.

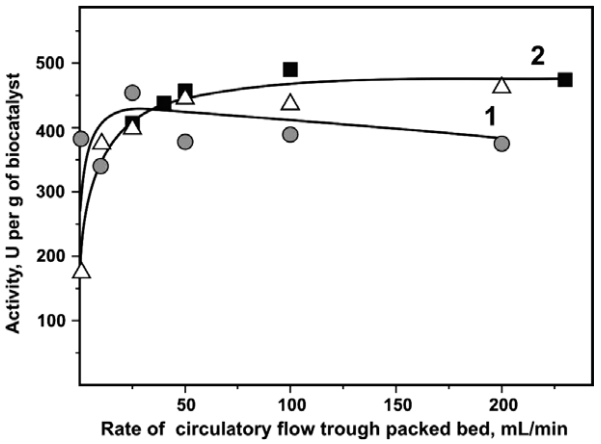

Figure 3. Dependence of the observed biocatalyst activity in a packed-bed reactor on the circulatory flow rate of substrate solution through the biocatalyst bed. (1) The biocatalyst is 0.5 cm (●); (2) the bed height are 2.0 cm (■) and 5.7 cm (△). The activity was measured under the standard conditions. The biocatalyst was prepared by the adsorption of GA on small Sibunit granules (∅ 0.2–1 mm).

Table 4. Thermal inactivation constants of glucoamylase immobilized on the carbon supports

T, °C	Sibunit $a = 10.0$ mg/g		Bulk CFC $a = 8.2$ mg/g		Activated carbon $a = 4.6$ mg/g	
	k_{in}^{E}, min^{-1}	$t_{1/2}$	k_{in}^{E}, min^{-1}	$t_{1/2}$	k_{in}^{E}, min^{-1}	$t_{1/2}$
65	3.4×10^{-3}	3.4 h	7.7×10^{-3}	1.5 h	1.4×10^{-2}	49 min
70	7.9×10^{-3}	1.5 h	3.1×10^{-2}	22 min	6.6×10^{-2}	10 min
75	1.9×10^{-2}	36 min	6.5×10^{-2}	11 min	1.4×10^{-1}	5 min
80	3.6×10^{-2}	19 min	Unstable		Unstable	

Then, the size of the support granules was selected in such a way that the observed hydrolysis rate did not depend on the granule size. This way the restriction by the internal diffusion was overcome. For glucoamylase immobilized on Sibunit, the dextrin hydrolysis rate was found to decrease when the size of the support granules exceeded 1 mm (Fig. 4). Meanwhile, the activity of the biocatalyst prepared using support granules smaller than 1 mm was constant and relatively high (Fig. 4). Hence, for the biocatalyst with the granule size larger than 1 mm dextrin diffusion inside the pore space of the granules was the rate-limiting stage of the starch saccharification process. The biocatalysts prepared using small granules of the support worked in the kinetic regime without diffusion limitations.

Under the conditions for kinetic regime, the temperature coefficients of dextrin hydrolysis were found to be the same equal to 1.6 for both immobilized glucoamylase and the soluble enzyme. This was an additional confirmation of the fact that the biocatalyst 'Glucoamylase on Sibunit' worked in the kinetic region.

The investigation of the reaction kinetics in the Michaelis–Menten coordinates for soluble and immobilized glucoamylase showed that the biocatalyst 'Glucoamylase on Sibunit' exhibited a high activity in a wide range of dextrin concentrations from 10 to 35 wt/vol %, whereas a more complicated kinetic dependence was obtained for the soluble enzyme (Fig. 5). As is seen from Figure 5, after immobilization, glucoamylase retained ~20% of the activity of the soluble enzyme. The dissociation constant of the enzyme–substrate complex, K_S, increased after immobilization. Assuming that the average molecular weight of dextrin is 3–5 kDa, these constants were estimated to be 1.7×10^{-4} M and 2.7×10^{-4} M (0.6 and 1.0 wt/vol %) for soluble and immobilized glucoamylase, respectively.

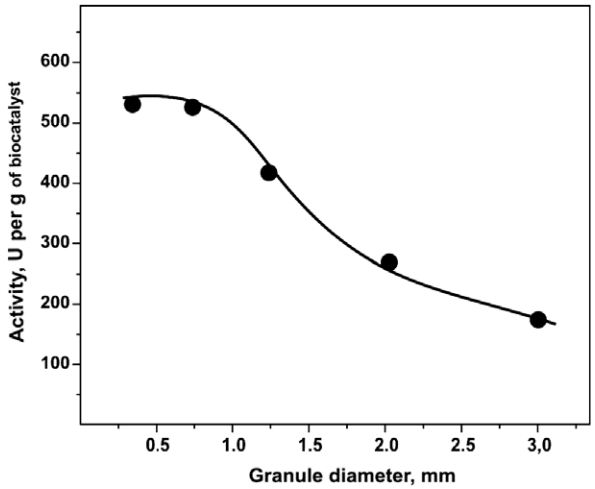

Figure 4. Dependence of the biocatalyst activity on the Sibunit support granules size (mm). The reaction was carried out under the standard conditions. The rate of the substrate solution circulation through the biocatalyst bed was 30 mL/min.

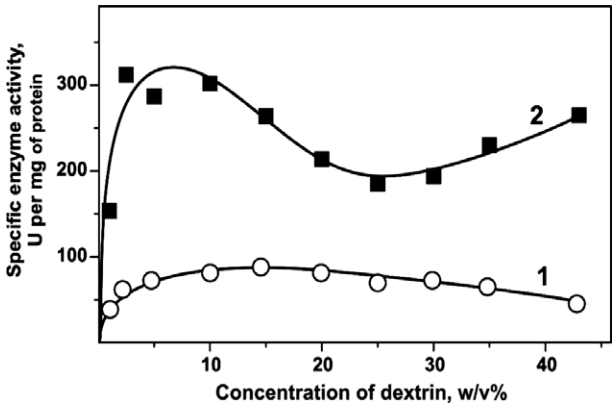

Figure 5. Dependence of the initial specific activity of soluble and immobilized glucoamylase on the initial concentration of potato dextrin. (1) 'Glucoamylase on Sibunit'. (2) Glucoawamorin® in solution. The activity was measured at 50 °C and pH 4.6.

It was found that the optimum pH for the glucoamylase activity did not change after immobilization on the carbon support and remained at pH 4.5–5.0 (results not shown). The only difference was that the immobilized glucoamylase showed maximum activity in a narrower pH range, whereas for the enzyme in solution, the optimum pH range was pH 3.0–6.0 (results not shown). This fact can be explained by preferential adsorption of glucoamylase from the solutions of the Glucoawamorin®, which also contains ballast proteins and accompanying enzymes. Thus, glucoamylase homogeneity and its degree of purification improve after adsorption. Indeed, the investigation showed that the specific enzyme activity in the solution over the Sibunit support (after adsorption) was ~1.5 times lower than the specific activity before adsorption, indicating preferential adsorption of glucoamylase molecules. Glucoamylase in a relatively pure enzyme sample from different companies ('BDH', 'Sigma', and 'Serva') is known to have a relatively narrow optimum range of pH 4.5–4.6.

The investigation of the temperature optimum for the dextrin hydrolysis reaction showed that glucoamylase both in solution and in the immobilized state has a relatively narrow temperature optimum, showing the highest activity at 65–70 °C (results not shown).

As mentioned above, the most important characteristic for the industrial application of a biocatalyst is its stability. The glucoamylase thermal stability increased by more than an order of magnitude after its immobilization by adsorption on the surface of the carbon supports evaluated (Table 3). Investigation of the thermal inactivation of immobilized glucoamylase at 80 °C showed that in the presence of the substrate (dextrin) the biocatalyst 'Glucoamylase on Sibunit' was substantially (by an order of magnitude) more stable than in

the buffer solution. For example, in the 37 wt/vol % dextrin solution, the inactivation constant of the immobilized glucoamylase at 80 °C was equal to 3×10^{-3} min^{-1}, whereas in the buffer solution it was equal to 4.0×10^{-2} min^{-1} (Fig. 6). The increase of the substrate concentration from 1 to 50 wt/vol % resulted in nearly linear growth of the thermal stability of the 'Glucoamylase on Sibunit' (Fig. 7). As is seen from Figure 7, in the 53 wt/vol % dextrin solution, the immobilized glucoamylase completely preserved its initial activity. Further increase of the concentration of dextrin prepared by starch hydrolysis is practically impossible because it is difficult to carry out the liquefaction of a starch suspension with the concentration of dry-weight substances more than 50 wt/vol %.

The investigation of the operational stability of the 'Glucoamylase on Sibunit' showed stepwise inactivation of the biocatalyst under conditions simulating the starch saccharification technological process (60 °C, 32 wt/vol % dextrin) (Fig. 8). The biocatalyst inactivation half-time under these conditions exceeded 350 h. A comparison of the characteristics of the biocatalyst prepared in this study with the results known from the literature showed that the main parameters of the biocatalyst 'Glucoamylase on Sibunit' exceeded those of the best reported analog[4] as well as that of the Corning Glass biocatalyst tested on the pilot plant scale[3] (Table 5). The calculations show that the total productivity of the 'Glucoamylase on Sibunit' biocatalyst after operation for $2 \times t_{1/2}$ (700 h) at a productivity of 7.6 kg h^{-1} kg$_{cat}^{-1}$ in a novel type of immersed vortex reactor will be 5.3 tons of glucose per kg of the biocatalyst.

We have also compared different types of reactors, including an immersed vortex reactor (IVR), in the process of dextrin hydrolysis. As mentioned above, the main attention in the IVR design was devoted to the substantial intensification of mass transfer of the substrate from the liquid phase to the enzyme immobilized on a solid porous support and elimination of stagnant zones. The laboratory model of the developed immersed vortex reactor was tested in heterogeneous dextrin hydrolysis by the biocatalyst 'Glucoamylase on Sibunit', and optimal operational conditions were determined for this reactor. It was shown that the observed dextrin hydrolysis rates practically did not change when the reactor body's rotation rate was increased above 300 rpm (Fig. 9). It means that at this rotation rate the external diffusion limitations were minimized. It was also found that higher body rotation rates were required to overcome the external diffusion limitations for the higher active biocatalyst. For instance, the body rotation rates were higher by ~100–200 rpm for the more active biocatalysts than for the less active ones

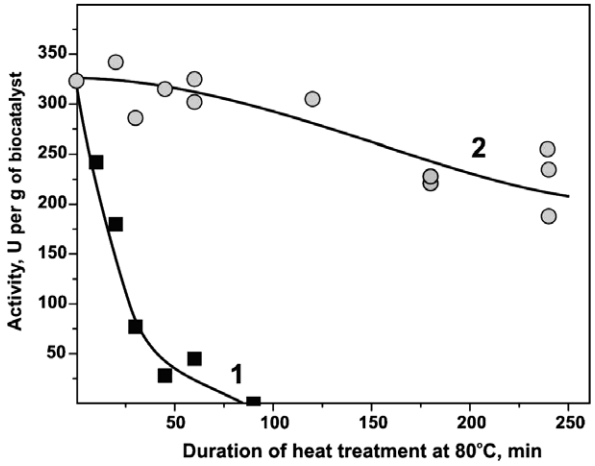

Figure 6. Changes in the activity of immobilized glucoamylase at 80 °C: (1) Buffer solution; (2) 37 wt/vol % solution of potato dextrin. The residual activity was measured under the standard conditions.

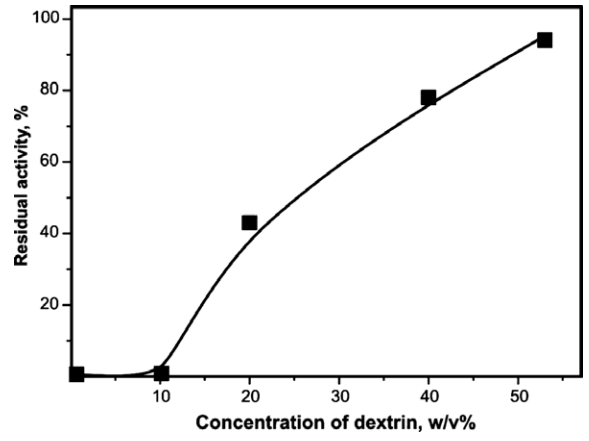

Figure 7. Dependence of the residual GA activity after heating the biocatalyst at 80 °C for 2 h on the dextrin concentration. The residual activity was measured under the standard conditions.

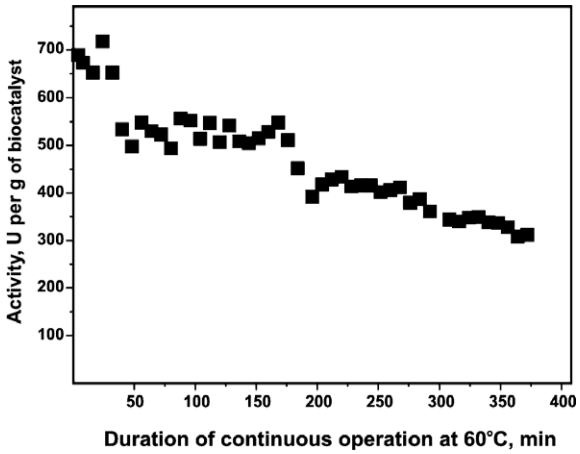

Figure 8. Dependence of the biocatalyst activity on operation time in the starch saccharification process (operational stability of the biocatalyst). *The activity was tested under the following conditions*: 60 °C; 32 wt/vol % solution of potato dextrin; the rate of the substrate flow through the biocatalyst bed was 20 mL/min.

Table 5. Comparative characteristics of the biocatalyst 'Glucoamylase on Sibunit' with literature data

Properties of the biocatalyst	'Glucoamylase on Sibunit'	'Glucoamylase on polystyrene'[4]	CORNING GLASS biocatalyst 'Glucoamylase on silica'[3]
Inactivation half-time, h	350 (60 °C)	300 (50 °C)	150 (60 °C) 7.5 (70 °C)
Activity, μmol min^{-1} g^{-1}	700	500	12
Specific enzyme activity, μmol min^{-1} mg^{-1}	60	15	110
Reactor	Vortex	Fluidized-bed	Packed-bed

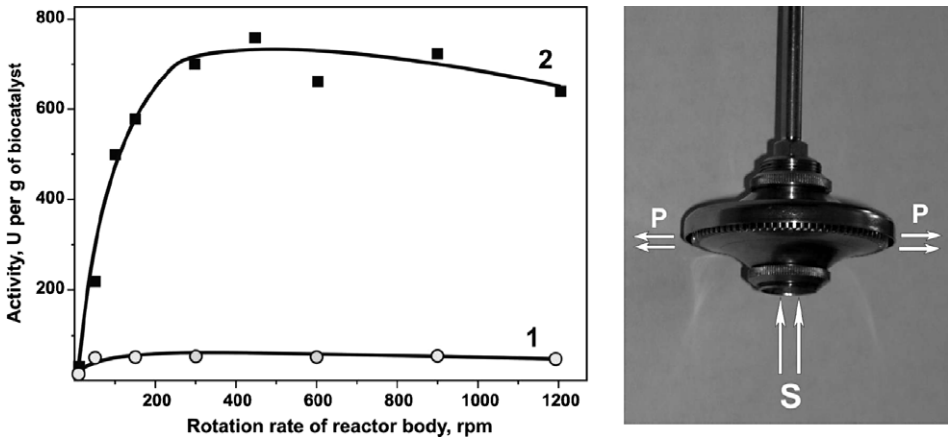

Figure 9. Effect of IVR body rotation rate on the observed activity of the biocatalyst 'Glucoamylase ion Sibunit': (1) Granules with diameter ∅ = 2–3 mm; (2) granules with diameter ∅ = 0.2–1 mm. The activity was measured under the standard conditions.

(Fig. 9). As noted above, the dextrin hydrolysis process was carried out deeply in the internal diffusion region. So, the reaction rate observed in IVR increased by an order of magnitude when the size of Sibunit granules was decreased from 3 to 0.2 mm (Fig. 9).

The efficiency of the IVR operation in dextrin hydrolysis was estimated by comparing the reaction rates observed in a vortex reactor and in a packed-bed reactor (PBR) under conditions with minimum diffusion limitations. The most active biocatalyst 'Glucoamylase on Sibunit' prepared by glucoamylase adsorption on mesoporous fine-disperse Sibunit was used in these experiments. It was found that in PBR the density of the biocatalyst bed and the hydrodynamic resistance increased strongly when the bed height ($h > 6$ cm) and the circulation rate (>200 mL/min) were increased. The comparison of the dextrin hydrolysis rates in kinetic region for the two types of reactors showed that the reaction rate and, as a consequence, the reactor productivity were higher by 20–50% in the vortex reactor than in the packed-bed reactor (compare maximal observed biocatalytical activity in Figs. 9 and 3).

The modern technology used for starch conversion into food sweeteners (treacle, glucose syrups) is based on enzymatic (rather than acid) hydrolysis[2] and includes the following stages: (1) gelatinization of 40% starch suspension at 100–110 °C; (2) hydrolysis by the enzymes, α-amylase and pullunase at 60 °C and pH 6.5 for 10–100 min (homogeneous stage); (3) filtration, ion exchange; (4) enzymatic hydrolysis of dextrin by glucoamylase at 55–60 °C and pH 4.5 for 24–96 h (homogeneous stage); (5) enzyme inactivation and extraction. The lab-scale results obtained in this study suggest the following changes to this technology: Stages (1) and (2) can be combined due to the high thermal stability of the used α-amylase from Amylosubtilin®, and hydrolysis can be carried out at higher temperature ~90 °C in aqueous solutions at pH 5.5–6.0. Stage (4) can be carried out in a heterogeneous regime using the biocatalyst 'Glucoamylase on Sibunit' and the immersed vortex reactor in 2–6 h (instead of 24–96 h). It should be noted that the carbohydrate composition of starch treacle can be easily regulated by simple stopping of the reactor and its removal from the reaction mixture. Due to the multiple use of glucoamylase and consequent lack of protein impurities, some steps of the final product purification in stages (3) and (5) are significantly simplified.

3.4. Conclusions

The data prove that the pore structure of the carbon supports that were studied, rather than their hydrophobic–hydrophilic properties, plays the key role in improving the stability of the immobilized glucoamylase. Mesopores matching the size of hydrated enzyme molecules and providing multipoint enzyme binding with the support are required for the preparation of highly stable heterogeneous biocatalysts.

It has been shown that after the immobilization of glucoamylase from Glucoawamorin® on the mesoporous carbon support, Sibunit, some kinetic characteristics of the enzyme, including the constant of enzyme affinity to the substrate (dextrin with molecular weight 3–5 kDa), pH- and temperature optima of the enzymatic reaction are practically unchanged. Meanwhile, the thermal stability of the enzyme considerably improves in comparison with the enzyme in solution. It has been found that the thermal stability of the immobilized enzyme is significantly affected by the substrate, so that an increase of the dextrin concentration from 1 to 50 wt/vol % results in a linear growth of the thermal stability of the immobilized glucoamylase. It has been found that the thermal stability of the immobilized enzyme in a 53 wt/vol % dextrin solution is $\geqslant 10^5$ times better than that of the enzyme in solution. The operational stability of the biocatalyst 'Glucoamylase on Sibunit' prepared by Glucoawamorin® immobilization on Sibunit is relatively high. Under model technological conditions of dextrin hydrolysis at 32 wt/vol % concentration at 60 °C, the inactivation time exceeded 350 h (30 working days).

The immersed vortex reactor (IVR) suggested in this study significantly improves the productivity of heterogeneous dextrin hydrolysis, which is controlled both by the internal and the external diffusion of the substrate to the immobilized glucoamylase. The external diffusion limitations were completely overcome at a reactor body rotation rate above 300 rpm, whereas the internal diffusion limitations could be minimized by decreasing the size of the support granules below 1 mm. Under these conditions the maximum productivity of the IVR exceeded that of the packed-bed reactor by a factor of 1.2–1.5.

A technological scheme for the production of starch treacle with the dextrin hydrolysis stage carried out heterogeneously using the 'Glucoamylase on Sibunit' biocatalyst and an immersed vortex reactor has been constructed and tested on a laboratory scale. The advantages of the new technological scheme are significantly accelerated dextrin hydrolysis, energy and resource saving and high quality of the final products due to the lack of protein impurities and easily regulated carbohydrate composition of the treacle.

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