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Macrostructured carbonized ceramics as adsorbents for immobilization of glucoamylase

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Abstract

Macrostructured carbonized ceramics have been synthesized and studied for the immobilization of glucoamylase. Carbon layer of different morphology, in particular of filamentous one, has been obtained on the surface of honeycomb monoliths and foam ceramic supports. The effects of morphology of surface carbon on adsorption and biocatalytical properties of immobilized glucoamylase, namely, on the stability and the activity of biocatalyst in continuous starch hydrolysis, have been investigated.

Keywords: Foam; Honeycomb monolith; Filamentous carbon; Adsorptive immobilization; Glucoamylase

1. Introduction

Immobilized enzymes, as well as immobilized bacterial cells are at the heart of innovative biotechnological processes as alternatives to traditional chemical technologies and hybrid processes including biochemical stages to substitute of some chemical ones. The main advantage of immobilization is the combination of unique biocatalytic properties of enzymes with heterogeneity that makes it possible to reuse them in continuous flow reactors. The *heterogeneous mode* in industrial-scale biotechnological processes is, undoubtedly, the most attractive because results in reducing the production cost. For example, rearrangement of the enzymatic process for starch hydrolysis into the heterogeneous mode leads to reduction of the operation expenses by 33% for production of glucose syrups [1].

The earliest pilot-scale process of glucose production based on immobilized glucoamylase via covalent binding on silica was realized by Corning Glass (USA) in 1970s and 1980s. Majority of patents on immobilization of amylolytic enzymes, in particular glucoamylase, on various inorganic supports including carbon-containing materials also were applied for these years, activated carbon being predominant [2–4]. Studies on immobilization of glucoamylase are in progress now [5,6]. *Inorganic supports* for enzyme inmmobilization are of great interest because of their durability, and high mechanical strength for usage in packed-bed or fluidized-bed bioreactors, and relatively low cost. In addition, immobilization of enzymes by *adsorption* is economically feasible and attractive. If inorganic supports possess macrostructure such as the honeycomb monoliths and foam-like materials, the diffusion limitations of substrate transport toward biocatalyst are obviously minimized, and efficiency of process could be significantly run up.

In the present study, the adsorptive immobilization of glucoamylase was carried out on carbon-containing

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inorganic supports based on macrostructured ceramics coated by carbon with different morphology. Systematic studies were focused on biocatalytic properties (activity and stability) of the heterogeneous biocatalysts obtained.

2. Experimental

Aluminosilicate and cordierite honeycomb monoliths as well as aluminosilicate foam were used as macrostructured ceramic supports for glucoamylase immobilization. Honeycomb monoliths based on clay, natural talc and aluminum hydroxide were prepared by extruding through a specially designed die. When dried in air, they had the following geometrical parameters: cylindrical monoliths of 20 mm diameter and 20 mm length with 54 square channels $2 \times 2\,mm^2$ in size and 0.5 mm channel wall. Some of monolith supports were calcined at 900 °C, the monolith shrinkage being ca. 4% (support H1). Another part of supports was calcined at 1200 °C (support H2) that resulted in 13.5% decrease in the monolith dimensions due to sintering. Aluminosilicate foams (support F) were used as cylinders of 10 mm diameter and 40 mm length. Average dimension of rounded inlets and wall thickness were 0.7–2 and 0.65–1 mm, respectively. Porosity of foam material was about 75–80%.

Two methods were used for synthesis of carbon layer on the surface of macrostructured ceramic supports. The graphitized carbon layer was synthesized on the surface of monolithic support H1 by thermal decomposition of the sucrose impregnated in H_2 flow at 500 °C for 2 h. The catalytic filamentous carbon (CFC) layers of different morphology were synthesized on the surface of monolithic (H1, H2) and foam (F) supports by catalytic pyrolysis of a propane–butane mixture over nickel metal particles obtained by reduction of $(NiOH)_2CO_3$ supported on the outer surface of ceramics by homogeneous precipitation [7]. Catalytic pyrolysis of the propane–butane mixture was conducted at 500 °C. Main parameters of the macrostructured carbon-containing supports are given in Table 1. Graphite (S_{BET} = $8.2\,m^2/g$), carbonized soot of sibunit type (S_{BET} = $220\,m^2/g$) and bulk catalytic filamentous carbon (CFC, $S_{BET} = 130\,m^2/g$) were used for comparative studies.

Specific surface area (S_{BET}) of the prepared adsorbents was measured by thermal desorption of argon. Pore size distribution of the supports was determined by mercury porosimetry using an AUTO-PORE 9200 device. Phase composition of the supports was studied by recording XRD patterns using a diffractometer D-8 (Bruker) in Cu Kα monochromited irradiation by point-by-point scanning at the 2θ angle range between 5° and 70° and accumulation time 8–20 s. The recorded XRD patterns were interpreted using the PCPDF database. Electron microscopic studies of the surface carbon layer morphology were carried out using a digital image output-scanning microscope REM-100U. Specimens to be studied were prepared according to the following procedure: supports were fixed on aluminum foil by special conducting glue, and then covered with a gold layer in a VUP-4 apparatus. The bars on the micrographs correspond to the distance expressed in μm.

Adsorption of glucoamylase on honeycomb monoliths and ceramic foams was performed under dynamic conditions by slow circulation of enzyme solution at ambient temperature for 24 h. After immobilization procedure, the biocatalysts obtained were washing repeatedly by buffer. The adsorption was estimated from the difference between the protein amount in the solution before and after completion of the adsorption run and expressed as mg of protein per 1 g of support or per $1\,m^2$ of the carbon surface.

Table 1
Characteristics of macrostructured carbon-containing adsorbents

Legend of adsorbents	Support	Carbon content (wt.%)	Specific surface area (m^2/g)	The specific surface area of the isolated carbon layer (m^2/g carbon)
GLC/H1	H1	10.1	27	50
FLC/H1	H1	4.8	49	400
HLC/H2	H2	0.6	0.8	150
FLC/F	F	2.4	9.7	350

Specific dye Coomassie G-250 was used to determine the protein concentration in the solution according to the modified procedure described elsewhere [8], bovine serum albumin (Serva) being used as the reference.

The enzymatic activity of *soluble* and *immobilized* glucoamylase was determined under the following conditions: 0.05 M acetate buffer at pH 4.6; 1% (w/v) buffer solution of starch. The reaction temperature was varied from 25 to 65 °C. Overall g*lucoamylase* activity was measured in reaction of starch hydrolysis, the rate of glucose generation being measured in the course of the reaction. Glucose concentration was determined spectrophotometrically by glucose oxidase (GO) method described in [9]. The GO activity in solution used for analysis was calibrated twice a day before determination of glucose concentration in starch, the standard glucose concentrations being used. The specific enzymatic activity of glucoamylase was expressed as μmol of produced glucose per min per 1 mg of protein (μmol/min/mg of protein or Units/mg of protein). The experimental error did not exceed 15%.

The rate of starch hydrolysis by immobilized glucoamylase was measured using a circulation installation including a peristaltic pump Zalimp pp 1-05 and thermostated column reactor. The 1% (w/v) starch solution (10–20 ml) was circulated through the reactor at the flow rate of 7–72 ml/min. The aliquots of reaction medium (0.05–0.1 ml) were periodically drawn and analyzed for glucose concentration by method described in [9].

The long-term stability was determined when immobilized glucoamylase was stored in buffer solutions (pH 4.6) at ambient temperature. For these experiments granulated biocatalysts were studied. The activity was measured under intensive shaking of biocatalysts in starch solution at 20 °C for 1 h. First, the residual activity was measured at two to four times a week and then at 2- or 3-month intervals. The loss of enzymatic activity was estimated as % of initial activity retained.

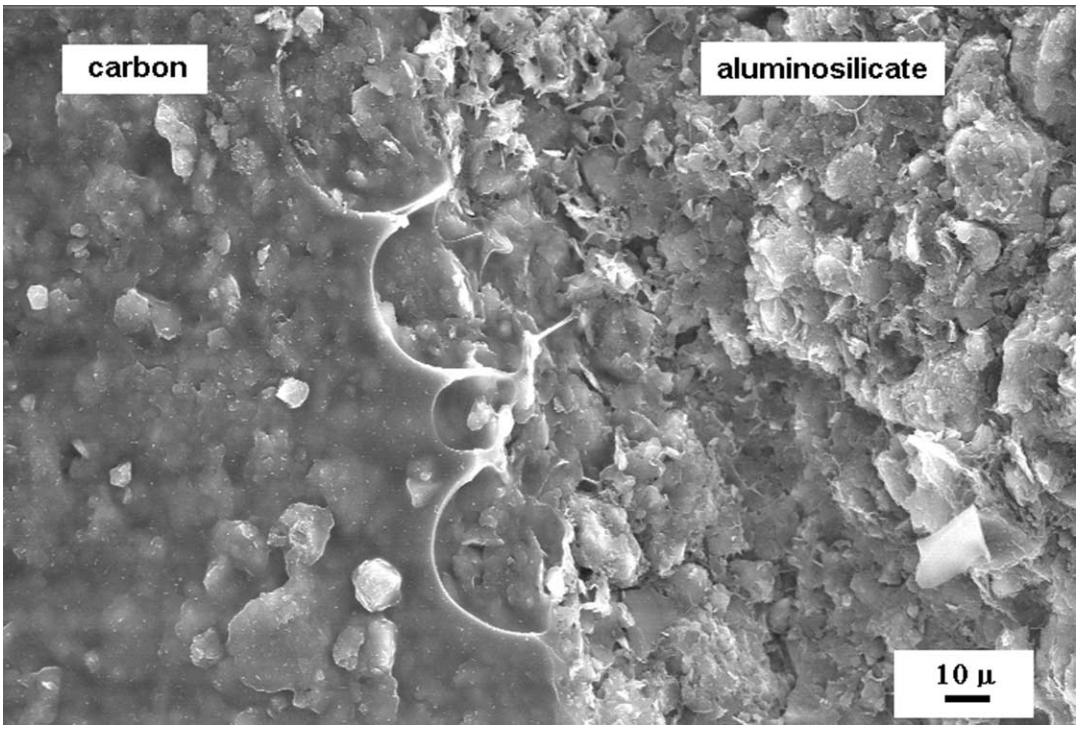

Fig. 1. Electron microscopic image of the surface of aluminosilicate support (H1) covered by GLC layer.

3. Results and discussion

Characterization of ceramic supports. The following parameters of monolithic support H1 calcined at 900 °C were determined: specific surface area 24 m^2/g; pore volume 0.25 cm^3/g; average pore radius 840 Å. XRD data showed that talc was the predominant crystal phase in H1. The other phases $2MgO{\cdot}Al_2O_3{\cdot}5SiO_2$ (cordierite), α-SiO_2 (quartz), $3Al_2O_3{\cdot}2SiO_2$ (mullite) and $Al_2O_3{\cdot}SiO_2$ (enstatite) were detected in small amounts.

Calcination of the monolithic support at 1200 °C (support H2) caused sintering and crystallization resulting in a decrease of the specific surface area to 0.13 m^2/g at the pore volume equal to 0.08 ml/g and in an increase of the average pore radius to 5 μm. Cordierite was the predominant crystal phase. Phases of quartz, mullite and enstatite were detected in trace amounts.

The specific surface area of ceramic foam (support F) was 0.12 m^2/g. XRD studies of this support revealed that predominant crystal phases were α-SiO_2, α-Al_2O_3 and $3Al_2O_3{\cdot}2SiO_2$, an amorphous phase characterized by a wide peak at the 2θ range of 18–25 and cordierite trace being also detected.

Characterization of macrostructured carbon-containing adsorbents. Specific surface area of macrostructured carbon-containing adsorbents in study (Table 1) depended strongly on morphology of the carbon layer synthesized on the surface of ceramics. That is why, the adsorbents were divided into three groups according to the morphology of carbon layer. Graphite-like carbon (GLC) was prepared on the surface of H1 support (Fig. 1). Fur-like carbon (FLC) formed by strongly interlaced thin carbon filaments was obtained on H1 and F supports (Fig. 2). Hair-like carbon (HLC) was synthesized on the surface of H2 support (Fig. 3). The specific surface area of the isolated carbon layer was estimated taking into account the values of specific surface areas of the non-carbonized and carbonized supports and carbon content (wt.%). The specific surface area of carbon layer on GLC/H1 adsorbent was equal to ca. 50 m^2/g of carbon (Table 1). In the case of FLC/H1

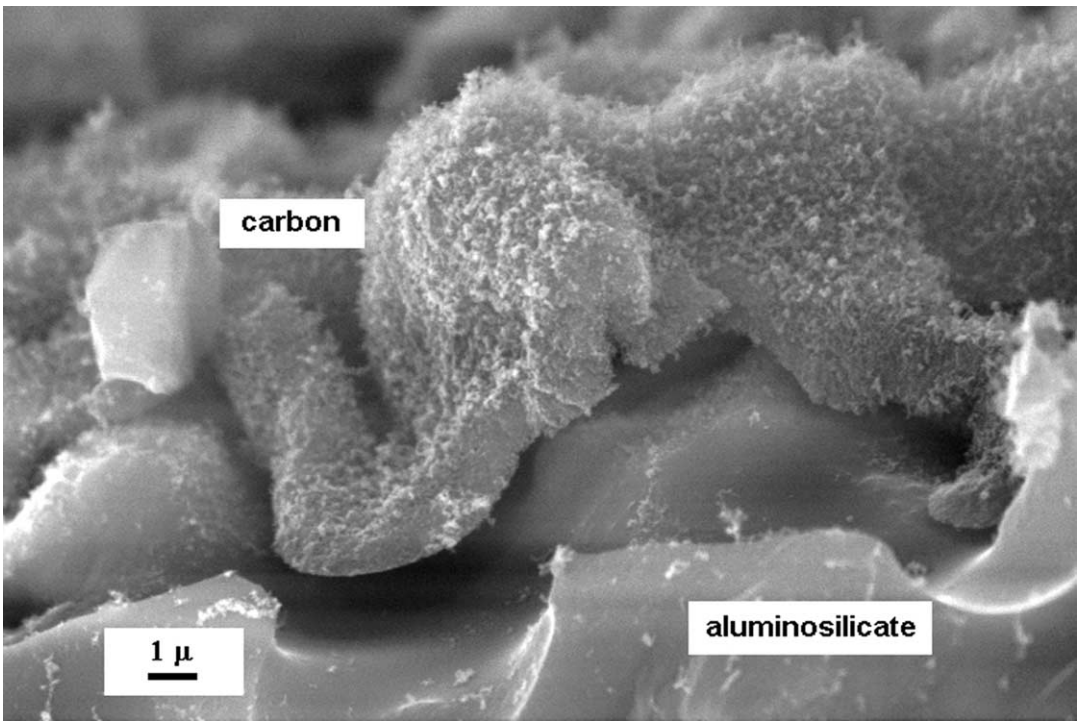

Fig. 2. Electron microscopic image of the surface of aluminosilicate support (F) covered by FLC layer.

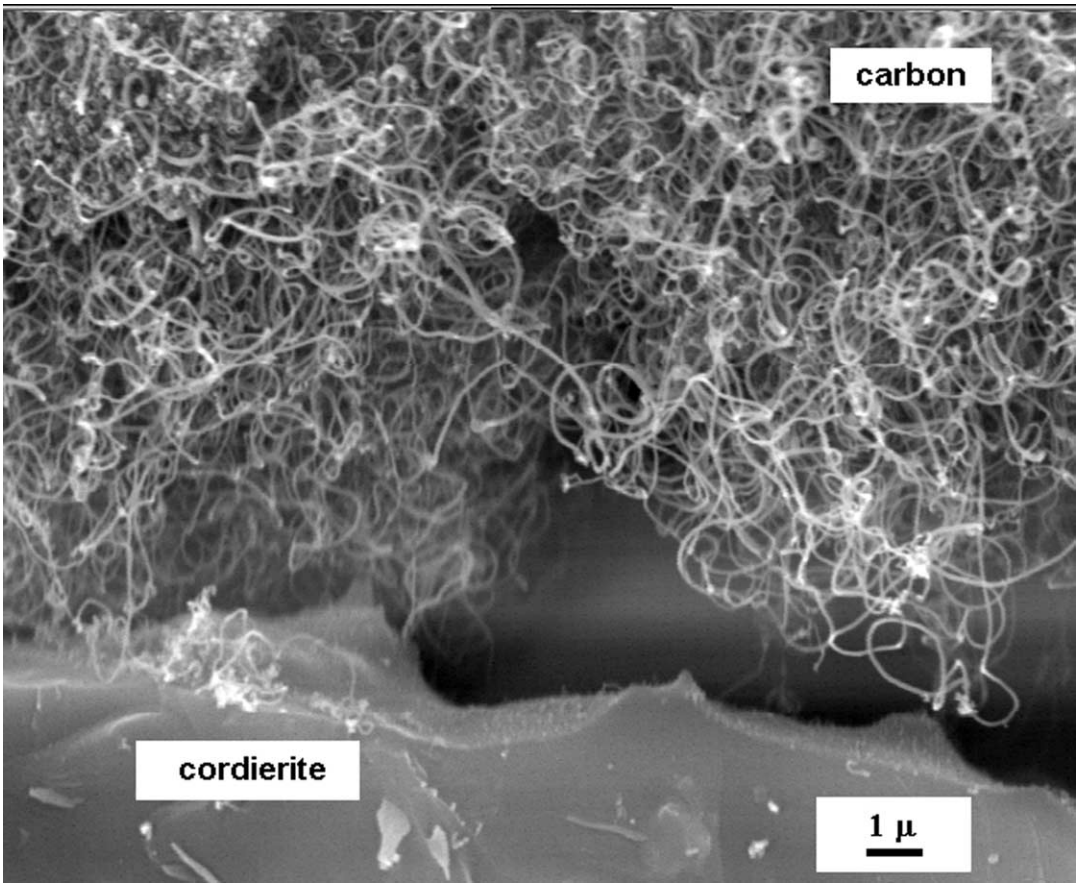

Fig. 3. Electron microscopic image of the surface of cordierite support (H2) covered by HLC layer.

and FLC/F adsorbents coated by filamentous carbon, the specific surface area of the carbon layer was ca. 400 m^2/g of carbon (Table 1). When the carbon layer was formed by thick and long carbon filaments like as for HLC/H2 adsorbent, its specific surface area was ca. 150 m^2/g (Table 1) with pore size of 1 μm and larger one.

Adsorption of glucoamylase. Comparative analysis of isotherms of glucoamylase adsorption showed that the character of the enzyme adsorption depended considerably on chemical properties and surface morphology of the adsorbent used (Fig. 4a). The isotherm of glucoamylase adsorption on the surface of GLC/H1 adsorbent looked like the Langmuir isotherm with a typical plateau assigned to formation of the monolayer at ca. 2 mg/g (Fig. 4a). Note, that a similar adsorption isotherm form was observed for glucoamylase adsorbed on graphite. S-like adsorption isotherms were observed for the FLC/H1 and HLC/H2 adsorbents covered by filamentous carbon (Fig. 4a). Multi-layered adsorption of glucoamylase was observed at the high (>2 mg/ml) equilibrium protein concentration (Fig. 4). Since glucoamylase was adsorbed on the surface of carbon, the adsorption was recalculated to mg of protein per 1 m^2 of surface area of the carbon layer (Fig. 4b). Interestingly, that the HLC/H2 adsorbent was found to be the most efficient if to compare with other adsorbents in study. The desorption of glucoamylase from supports was detected by residual enzyme activity in starch solution. It was found that the amount of protein desorbed was more less in the case of CFC-containing adsorbents. Therefore, CFC-like carbon bound glycoamylase more tight then graphite-like one.

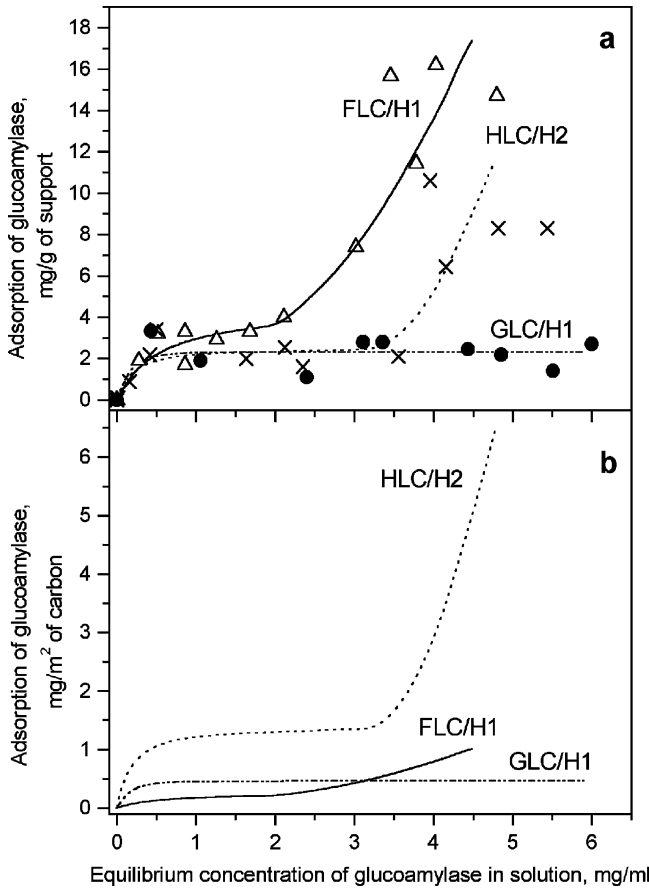

Fig. 4. Isotherms of glucoamylase adsorption on carbon-containing ceramic supports. (a) Adsorption was expressed as mg of protein per 1 g of support. (b) Adsorption was expressed as mg of protein per 1 m^2 of carbon. Adsorption was carried out at 20 °C for 24 h.

Stability of heterogeneous biocatalysts. Deactivation of immobilized glucoamylase (Fig. 5) depended on chemical origin of adsorbents and morphology of surface carbon layer. An abrupt decrease in the activity (down to 10–20% of the initial value) during the first months of storage was observed for *non-carbonized* H1 and H2 supports (Fig. 5a–c) and for graphite-like carbonaceous adsorbents such as GLC/H1, graphite, sibunit (Fig. 6c and d). In the case of bulk CFC, FLC/H1, FLC/F and HLC/H2 adsorbents, no significant change in their activity was observed during long period of time for 1 year (Fig. 5a, b and d). Thus, the morphology of carbon layer was a crucial factor affecting the stabilization of immobilized glucoamylase. It may be explained that, at first, the enzyme adsorption on CFC-containing surfaces was found to be tighter, and, at the second, the pores of CFC-like layer were more suitable in size to the protein globules.

Studies of *thermostability* of glucoamylase demonstrated that the stability was higher by an order of magnitude for the enzyme immobilized on filamentous carbon if to compare with soluble enzyme. The half-life time ($t_{1/2}$) for the enzyme inactivation at 65 °C was equal to 9.5 h for the glucoamylase immobilized on FLC/H1 and $t_{1/2}$ was no longer than 0.5 h for the soluble glucoamylase.

Activity of heterogeneous biocatalysts. Biocatalytic activity of the obtained biocatalysts was measured by varying of circulation rates (Fig. 6). As may be

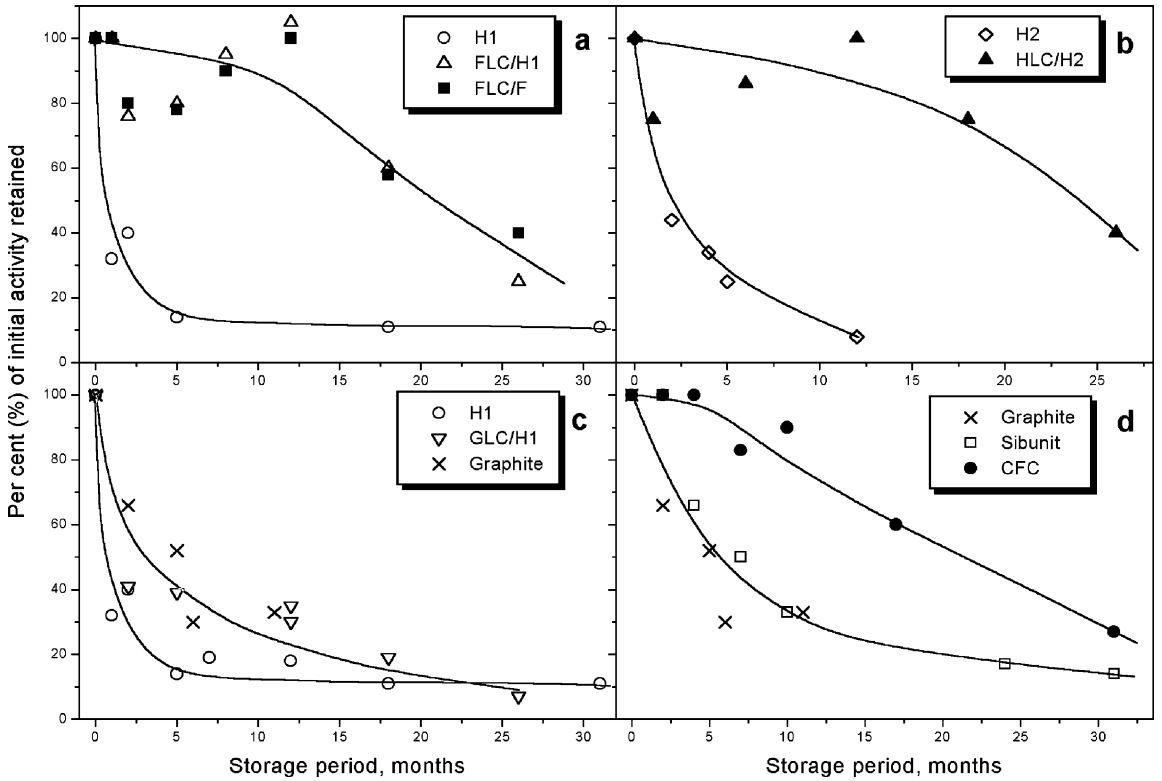

Fig. 5. Long-term stability of heterogeneous biocatalysts based on glucoamylase immobilized vs. time of storage at ambient temperature. Activity of glycoamylase immobilized was tested under intensive shaking of granulated (0.22 mm) biocatalysts in starch solution at 20 °C.

seen, honeycomb monoliths of FLC/H1 and HLC/H2 types differed strongly by specific enzymatic activity, the enzyme activity being observed to be 2-fold higher for HLC/H2 over the range of the feed flow rates under study (Fig. 6). So far as these adsorbents possessed the same geometry of monolith channels but differed by the morphology of carbon layer (see Figs. 2 and 3), this observation revealed the key role of the carbon layer morphology on the biocatalytical activity of glucoamylase immobilized. As mentioned above, compact carbon layer of FLC/H1 and FLC/F adsorbent (see Fig. 2) was characterized by smaller size of pores that probably resulted in limitations to transport of high-molecular starch through the carbon layer to glucoamylase immobilized. The specific enzyme activity of glucoamylase adsorbed on FLC/H1 and FLC/F-adsorbents with similar carbon layer morphology were close to each other (Fig. 6) in spite of significant difference in their channels geometry. Thus it was shown that starch hydrolysis was strongly controlled by starch diffusion inside surface carbon layer of biocatalyst studied.

4. Conclusions

1. *Adsorption properties of* carbon-containing ceramic supports were studied with respect to glucoamylase. Supports with a *catalytic filamentous carbon* layer on their surface were shown to be the most efficient adsorbents. The enzyme was the most tightly immobilized on the surface of these adsorbents. Adsorption of glucoamylase was shown to be multi-layered, the monolayer coating being ca. 2 mg/g of support.

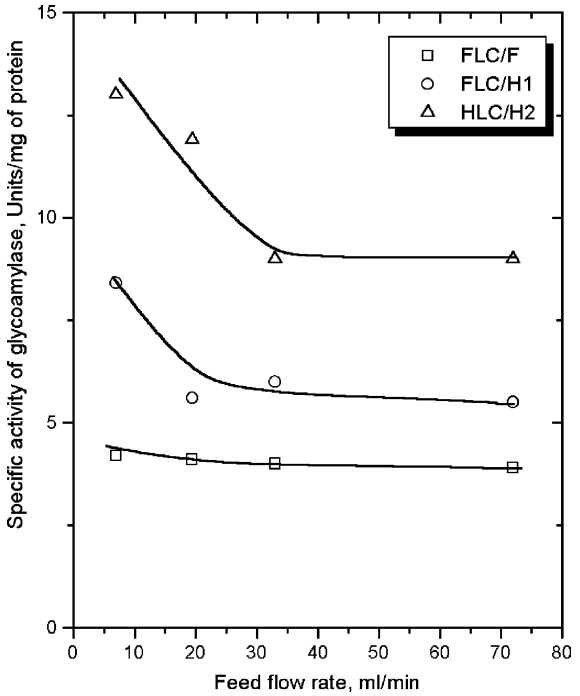

Fig. 6. Biocatalytic activity of heterogeneous biocatalysts in the reaction of starch hydrolysis vs. feed flow rate. Adsorption of glucoamylase on HLC/H2, FLC/H1, and FLC/F were 0.04, 0.14 and 0.17 mg/g, respectively. Activity of immobilized glycoamylase was tested at 50 °C.

2. In order to develop a *high-stable* heterogeneous biocatalyst for continuous starch hydrolysis, carbon layer on the supports for glucoamylase immobilization should be of filamentous morphology. If so, the stability of glucoamylase increased by an order of magnitude if to compare to that of the soluble enzyme. Immobilized glucoamylase retained its high biocatalytic activity during 1–1.5 year storage at ambient temperature.
3. Hydrolytic *activity* of heterogeneous biocatalysts was studied in the continuous flow reactors based on carbon-containing honeycomb monoliths and ceramic foams. *Diffusion* of starch inside of carbon surface layer was shown to be the limiting stage of the process of starch hydrolysis.

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