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LIPASE-MIMETIC ACTIVITY OF NANOPOROUS CARBON MATERIALS

The article discusses lipase-mimetic activity of nanoporous carbon materials (activated carbons of SKN and KAU type and their modified forms) in the reaction of p-nitrophenyl palmitate hydrolysis at pH 6.0–8.0 range. It was found out that oxidized forms of SKN_{ox} and KAU_{ox} exhibit their own catalytic activity, which is respectively 25 % and 12 % of the native enzyme activity. Using Candida rugose lipase physical immobilization on the surface of nanoporous carbon materials retains 30–50 % of its activity. Both carbon surface heteroatom dopping and its structure characteristics influence the activity of immobilized enzyme.

Keywords: nanoporous carbon materials, lipase, enzyme immobilization, catalytic hydrolysis, p-nitrophenyl palmitate.

Introduction

Enzymatic catalysis (biocatalysis) – a process that is widely used in many industries and has a number of advantages over traditional catalysis, such as high speed and selectivity, biodegradability and mild conditions of the reaction. One of industrially important enzyme groups is lipases (triacylglycerol hydrolases, EC 3.1.1.3) that accelerate the processes of ester hydrolysis in aqueous media and esterification / transesterification – in organic [1; 2]. This diversity of reactions, as well as high chemo-, region- and enantiospecificity makes them one of the most common enzymes used in food, pharmaceutical, textile, paper, and chemical industries [3–5]. Studies of the structure and functioning of lipolytic enzymes is particularly important because of their biological role – lipases are involved in the dissolution and fractionation of neutral fats in the human body. Currently this enzyme feature is widely used for wastewater treatment from waste oils/fats to reduce their environmental impact and parallel synthesis of fatty acids. Besides, conversion of oils/fats is usually held at high temperatures and pressure, while the enzymatic hydrolysis process is energy-efficient, which enables increase of the selectivity of the reaction and purity of the product [6–8].

Lipase consists of hydrophilic non-metallic reaction center (there is a sequence of amino acid residues (so-called "catalytic triad") Ser-His-Asp in most lipases) and hydrophobic α-helical oligopeptide "cap" ("domain"). Typically, the enzyme exist in a "closed" conformation – catalytic center is blocked by a hydrophobic "cap" and is inaccessible to the substrate [9]. A necessary condition for enzyme activation is the presence in the reaction solution of a hydrophobic surface that interacts with the "domain" and opens the access to the lipase's catalytic triad. This phenomenon, called "surface activation", is the defining characteristic of lipases [10; 11]. Obviously, the movement of the "domain" depends on the nature of the solvent [12] and pH of the microenvironment of the active center [13; 14]. Despite the complicated structure organization, lipases belong to the stable enzymes and retain their activity in the temperature range 40–60 °C and pH of 4.0 (pancreatic lipase) to 8.0 [3].

From an economic perspective, the use of immobilized enzymes is commercially desirable because they enable a) stabilization of the enzyme and reduction of the impact of denaturating agents on it; b) repeated or continuous use of the biocatalyst; c) isolation of the catalyst and reaction products from the reaction system; d) influence on the enzymatic reaction by changing the conditions of the process. Choosing the optimal carrier for enzyme immobilization is an important step in the creation of biocatalysts because enzyme activity significantly depends on the type of carrier and interactions with it. Such materials should have highly porous structure and surface area, high thermal and chemical stability, low cost. Furthermore, the carrier must have an affinity to the enzyme – specific qualitative and quantitative composition of surface chemistry that provides maximum activation of the enzyme and facilitates contact "enzyme substrate" [14].

To date, lipase has been successfully immobilized on aluminum oxide, silicon oxide, porous glass, sepharose, cellulose, zeolites, polyethylene, polypropylene, polystyrene, nylon, and also on activated carbon (AC) [15]. Activated carbon is resistant to bacterial, chemical and mechanical

effects, and does not swell in biological fluids (blood). In addition, these carriers have highly developed surface area that allows to create on the surface a variety of functional groups, which significantly increases the amount of immobilized enzyme and its resistance to environmental influences. Such characteristics are prerequisite for the creation of biocompatible and bioselective biologics and medications with a wide range of applications in the food and pharmaceutical industries and medicine.

Physical adsorption is a simple and convenient method of enzyme immobilization. This method has proved its efficiency when applied for lipase immobilization on mesoporous carbon material in continuous reactors. Mesopores create for the enzyme an "ampullaceous" space that prevents lipase desorption from AC surface and at the same time retains its activity [14]. Analysis of papers devoted to the study of the biocatalysts based on AC and lipase shows that the activity of created samples is higher than the activity of native enzyme [14; 16; 17]. This result is explained by the change of lipase conformation from "closed" to "open" during its immobilization on the carrier. However, this phenomenon may be related to the cumulative effect of the carrier and the enzyme

This work is devoted to the research of lipase-mitetic activity of nanoporous carbon materials (NCM) and their modified forms in comparison with the activity of native and immobilized enzyme in the reaction of p-nitrophenyl palmitate hydrolysis at different pH.

Materials and methods

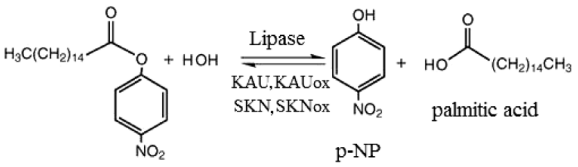
Lipase from Candida rugosa (Sigma, activity – 819 U/mg) with concentration of 0.1 mg/ml; p-nitrophenyl palmitate (Fluka); surfactants Triton X-100 (Aldrich); 2-propanol; 0.067 M phosphate buffer (PB) ($Na_2HPO_4 + KH_2PO_4$) with pH values of 6.0–8.0 were used in this work.

Nanoporous carbon materials from fruit stone (KAU) and synthetic nitrogen-containing (SKN) activated carbons were chosen as carriers for lipase. Modification of the carbons was performed by the oxidation with nitric acid. For this purpose, 5 g of NCM heated on the water bath (100 °C) with 25 % nitric acid (V = 37,5 ml) during 3–4 hours. After that NCM was washed with distilled water to pH 6–7, and then it was treated with 5 % solution of NH_4OH followed by boiling of the samples in distilled water. The operation was repeated 6–9 times to get colorless solution over the NCM (removing humic acids). Full transformation of oxidized samples in H-form was performed with of 3 % solution of HCl in volume ratio "NCM : acid" 1 : 3 with constant stirring overnight. Then coal was washed with distilled water to pH 6–7 and dried at a temperature of 100–105 °C.

The method of thermal adsorption/desorption of nitrogen at T = 298 K was used to study the structural and sorption characteristics of nanoporous carbon materials. The values of the pore volume (Vs, cm^3/g), pore diameter (d, nm), and specific surface area (SSA, m^2/g) were calculated from obtained isotherms. Qualitative and quantitative characterization of surface functional groups was performed by Böhm titration [18]. 50 ml of 0,05 M HCl, $NaHCO_3$, Na_2CO_3 and NaOH solutions was added to four samples (0.1 g) of activated carbon and stirred for 24 hours. Then the solution concentration above carbon samples was analysed. The number of oxygen-containing groups was determined by titration of the samples with 0.05 M HCl, given that NaOH solution neutralizes carboxylic, lactone and phenol group; Na_2CO_3 – carboxylic and lactone, while $NaHCO_3$ – only carboxylic.

Enzyme immobilization was performed using lipase adsorption method when enzyme solution (pH = 7.0) contacts with NCM (0.04 g) for 24 hours (T = 4–6 ºC). It is believed that within 24 hours the equilibrium between lipase in solution and on the carrier surface is established. After adsorption the solution was decanted and the NCM samples were washed with phosphate buffer (pH = 7.2, V = 10 ml).

Catalytic activity of nanoporous carbon materials, native and immobilized lipase was measured in the reaction of p-nitrophenyl palmitate (p-NPP) hydrolysis:



The emulsion of the substrate was obtained by mixing p-NPP and the surfactant Triton X-100 with 20 mL of 2-propanol and 200 ml PB with the necessary pH. The volume of added Triton X-100 was changed proportionally to the weight of p-NPP (substrate concentration increased within the interval 0.193–0.965 mg/L) to form stable in time emulsion. Catalyst (0.4 ml of lipase, or 0.04 g of pristine and oxidized NCM, or samples with immobilized enzyme) was added to 50 ml of substrate emulsion. The rate of hydrolysis of p-NPP in the reaction mixture was determined by the

accumulation of the colored reaction product – p-nitrophenol (p-NP) through periodic sampling of the reaction media. The reaction was carried out at room temperature for 2 hours at different pH values (6.0; 6.5; 7.0; 7.4; 8.0) and substrate concentration.

The solutions of p-nitrophenol with a concentration of 0.008 to 0.08 g/L were prepared, optical density was determined spectrophotometrically (λ = 400 nm, L = 0.2 cm). The data was used to build the calibration curve of absorbance – p-nitrophenol concentration (Fig. 1).

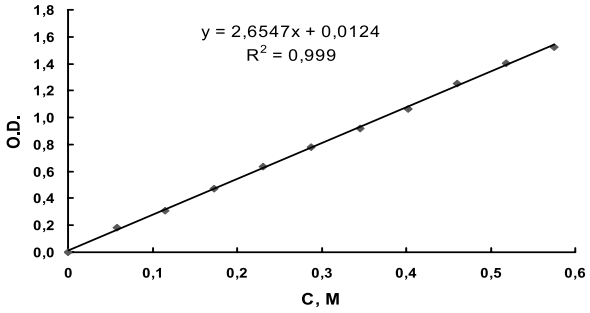


Fig. 1. Calibration curve of p-nitrophenol

To quantify and compare the lipase and lipase-mimetic activity of initial and oxidized NCM and the carriers with immobilized enzyme the equation of enzyme kinetics was used (Michaelis – Menten equation)

$$V = V_{\max} \frac{C}{K_m + C},$$

where V – the initial velocity of hydrolysis (mol/g•s); V_{max} – maximum speed of hydrolysis (mol/g•s); K_m – Michaelis constant; C – p-NPP concentration (mol/l).

For K_m determination the initial reaction rate at different concentrations of p-NNP was found. All other conditions of the experiment (pH, temperature, ionic composition, etc.) did not change. The results were used to build Lineweaver – Burke plot (1/V – 1/[C]), from which the values of K_m and V_{max} can be calculated by extrapolating the line to its intersection with the abscissa.

It is important to mention that the processes that take place at the interface "oil/water" do not obey the classical equation of Michaelis – Menten because the speed of this reaction depends not on the substrate concentration in the reaction solution, but on the amount of substrate on the interface [19]. So, to calculate kinetic constants according to the classical laws of enzyme kinetics it is necessary to increase not only the concentration of the substrate, but also the quantity of the surfactant so that their ratio remains constant. In this case, the catalytic process can be represented by the scheme (Fig. 2).

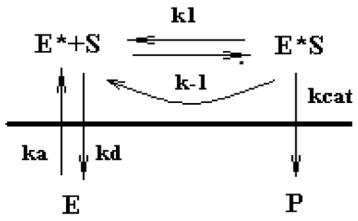


Fig. 2. Scheme of the interaction between the enzyme (*E*) and the substrate (*S*) on the interface

This mechanism of catalysis requires a certain time of activation of the enzyme on the interface (to go from E to E^*) with rate constant k_a, which was observed in the experiment. The formation of "enzyme-substrate" complex (E^*S) and the products of the reaction occurs at the interface and obeys classical Michaelis - Menten law. Such Michaelis constant is called "surface" (K_m^*). To simplify the quantitative interpretation of the data, the affinity constant K_{af} that has the inverse value to K_m^* was used.

Results and discussion

All known for today lipase can be conditionally classified into two categories – specific, activity which mainly depends on the nature of the substrate; and nonspecific catalytic ability which depends on the nature of the solvent and the pH of the reaction solution. Lipase *Candida rugosa* is a non-specific enzyme that acts on the majority ester bonds RC-(O)-OR with the formation of acids, alcohols, or new esters (esterification and transesterification reactions).

It was found that the length of the chain substrate affects on the activity of non-specific lipase, because the structure of the enzyme is L-shaped polypeptide tunnel, whose length is optimal for C_{12}-C_{18} chains [19; 20]. That is why for the study was chosen p-nitrophenyl palmitate with chain length of C_{15}. Hydrolysis reaction of p-NPP also depends on the nature of the solvent – catalysis can be carried out both in organic and in aqueous media using the buffer solutions [21]. However, there is a significant decrease in activity in organic media since: a) limited number of enzyme molecules is fully activated at the interface; another number is inactivated by organic solvent; b) uneven distribution of water molecules around the catalytic center, resulting in a "triad" does not reach its maximum of activity. Taking into account these factors activity of native lipase in phosphate buffer of pH, approaching to the enzyme optimum in biological media, in which operates lipase was examined in this work. It should be noted that a

number of organic substances (including alcohol) affect on the reaction rate of hydrolysis, which is associated with the growth of the reaction products solubility in aqueous media that can adsorb on the interface, reducing the enzyme activity [22].

The majority of lipases (pancreatic lipase is an exception) have optimum pH range from 6.5 to 7.5, which does not contradict with the obtained results (Fig. 3).

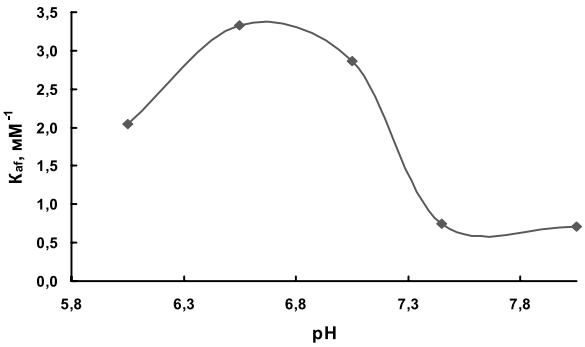


Fig. 3. Dependence of lipase activity in the p-NPP hydrolysis reaction on pH

Hydrolysis of ester by lipase active center consists of the following stages: (I) – serine residue activation by deprotonation under asparagine and histidine residues; (II) – attack the carbonyl group of the substrate via nucleophilic hydroxyl residue Ser and the formation of "acyl-enzyme" transition complex and oxianionic hole; (III) – deacylation transition state by water molecules [11; 14]. Hence lipase activity increases with increasing hydroxyl nucleophilicity of the serine residue, i.e., when the active center has a negative potential (neutral or alkaline). The data also confirmed by lipase *Candida rugosa* potentiometric titration, pointing to a sharp increase in negative potential of the enzyme in the pH range 5.0–8.0 [23].

electrostatic interaction mechanism, called "electrostatic catapult" [23].

Obviously, not only the presence of the interface, and also reproduction an optimal pH are necessary conditions for activating lipase and ensure its maximum activity. In the absence of surfactants and pH < 5 activity of *Candida rugosa* lipase cannot be measured by instrumental methods.

Structural and sorption parameters of the samples are presented in Table 1, which implies that the activated carbon KAU and SKN are *nanoporous carbon materials*, their pore diameter varies between 0.95–2.15 nm. Modification of samples leads to a slight increase in the pore diameter, pore volume and specific surface area that indicate partial destruction of NCM during oxidation. Carbon samples elemental analysis results show that during the oxidation number of oxygen atoms increases 35 times for KAU and twice – for SKN. Increasing the total nitrogen content can be associated with partial surface nitration.

Qualitative and quantitative composition of surface groups studied by Böhm titration method (Table 2). It was established that the total amount of acid groups is growing 20 times after sample oxidation and is almost the same for both types of NCM. However, carbon of SKN type has on the surface basic groups, due to the presence in its structure nitrogen-containing groups. Previously, it was found that nitrogen on the surface is in pyrrole and pyridine rings, heterocyclic and condensed polyaromatic systems, amino groups and N-oxides [24].

Enzyme-like properties of activated carbon samples in p-NPP hydrolysis was investigated for the first time and shown that pristine samples KAU and SKN not catalyze the reaction at a pH range 6.0–8.0. Probably substrate emulsion was adsorbed on the hydrophobic surface NCM that hindered the

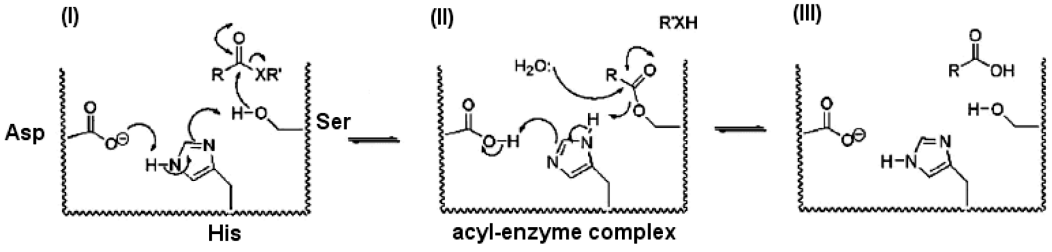


Fig. 4. Scheme of catalytic R-C(O)-XR' substrate hydrolysis by lipase [11]

The pH of the reaction solution affects on the diffusion of the reaction products from microenvironment of the lipase catalytic center. Newly created acid and alcohols which are deprotonated in neutral or alkaline, are disconnect from the negatively charged active center under the reaction [25]. However, the oxidatted samples exhibit lipase-mimetic properties at pH 7.4. For activated carbon SKN_{OX} K_{af} is 0.185 mM^{-1}; for KAU_{OX} – 0.087 mM^{-1}, that is only 25 % and 12 % respectively of the native enzyme activity under these conditions.

Table 1. **The elemental composition (mass. %) and structural-sorption parameters of NCM**

Type of AC	Elemental composition (mass. %)				V_s, cm^3/g	d_{pore}, nm	SSA, m^2/g
	C	H	O	N			
KAU	99.4	0.2	0.1	0.3	0.50	2.10	1070
KAU_{OX}	96.3	1.1	2.0	0.6	0.85	2.15	1850
SKN	91.2	1.0	6.5	1.3	0.75	0.95	1970
SKN_{OX}	85.8	0.9	11.3	2.0	0.8	1.30	2140

Table 2. **The content of functional surface groups of activated carbon samples**

Type of NCM	Groups, mM/g				
	carboxylic	lactonic	phenolic	acidic groups	basic groups
KAU	0.04	0.06	0.02	0.12	–
KAU_{OX}	0.84	0.20	1.39	2.43	–
SKN	0.08	0.02	0.02	0.12	0.12
SKN_{OX}	1.04	0.10	1.01	2.15	–

Lipase-mimetic ability of modified samples can be explained by the presence on their surface oxygen functional groups that caused: 1) reduction in substrate emulsion adsorption on the surface; 2) functional groups involved in the molecular mechanism of reaction like Asp, His, Ser residues of lipase catalytic "triad" (see Fig. 4); 3) promote efficient electrostatic interaction between reaction products and surfaces due to its negative charge at pH 7.4 ("electrostatic catapult"). It should be noted that the activity of nitrogen-containing samples SKN_{OX} exceeds twice for KAU_{OX} that may be associated with structural-sorption characteristics (SKN_{OX} surface area more than KAU_{OX} one), and with presence of nitrogen-containing surface groups.

The process of lipase immobilization by physical adsorption on activated carbon surfaces studied in the following works [14; 16; 17; 25; 26] and it was found that the activity of created biocatalysts depends on several factors:

1. Structural-sorption parameters of carrier. It is proved that for physical adsorption of the enzyme pore diameter should be 1.5 times more that the main axis of protein molecule. In the case of lipase, this value should be the value no less than 100 nm. It is obvious that lipase due to hydrophobic interactions adsorbed on the surface, but not in pores of KAU and SKN samples. As stated above, lipases have high affinity for hydrophobic surfaces; however, nanoporous structure of carbon materials creates diffusion barriers, largely reducing the activity of the enzyme on their surface. These conclusions are confirmed by obtained results (Fig. 5). Immobilized lipase is inactivated on the surface of samples KAU and SKN, because its activity missing in neutral pH and significantly reduced in an alkaline media.

2. Surface chemistry of carrier. Except dispersion interactions, between the enzyme molecule and the carrier, some specific interactions due to carrier functional groups appear. In this case, partial immobilization of an enzyme occurs that enables the substrate "free" hydrophobic "domain" adsorbed at the interface "oil/water". In addition, due to the interaction between carrier carboxyl groups and enzyme amino groups increases the number of lipase immobilized on the surface KAU_{OX} and SKN_{OX} [17; 25]. Under these conditions, the activity

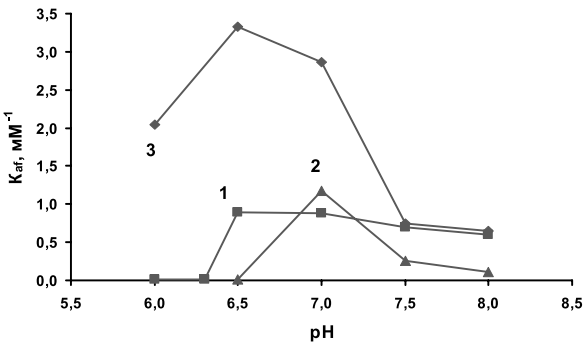


Fig. 5. The pH dependence of immobilized lipase activity on the surface (*1*) – KAU, (*2*) – SKN compared with native lipase (*3*)

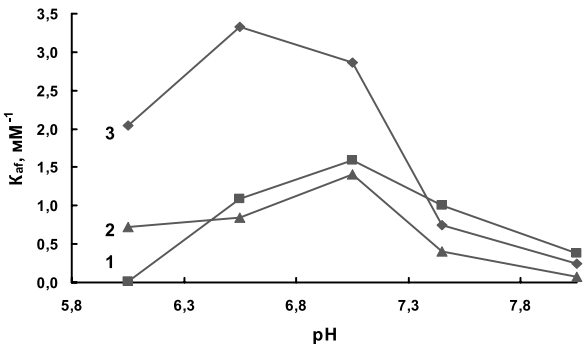


Fig. 6. The pH dependence of immobilized lipase activity on the surface (*1*) – KAU_{OX}, (*2*) – SKN_{OX} compared with native lipase (*3*)

of lipase persists in neutral pH, and in alkaline media activity immobilized sample exceeds that for native lipase (Fig. 6). Maximum activity is shifting towards alkali pH and it is 7.0.

Based on data, obtained for modified KAU_{OX} and SKN_{OX} can conclude that the increasing of immobilized lipase activity on the surface may be associated not only with the activation of the enzyme (lipase transition from a closed to an open conformation), but with synergistic (cumulative) effect of carrier and enzyme action. However, this effect is observed only for KAU_{OX} samples, and thus to preserve the activity of lipase affects pore size, not the value of its specific surface area and the presence of nitrogen-containing functional groups.

Conclusions

In this work the lipase-mimetic activity of nanoporous carbon materials, in particular, the activated carbons of SKN and KAU type, their modified (oxidized) forms in a hydrolysis reaction of p-nitrophenyl palmitate at different pH values was investigated. It was shown that pristine NCM samples have no lipase-mimetic activity in the pH range 6.0–8.0. However, oxidized samples SKN_{ox} and KAU_{ox} have catalytic ability in slightly alkaline media, which is 25 % and 12 % of the activity of native enzyme, respectively. Immobilization of enzyme on pristine CNM samples leads to its inactivation in neutral media, and at pH 7.4 lipase loses 46 % and 7 % of its activity on the SKN and KAU carrier respectively. The catalytic ability of created biocatalysts can be adjusted by the number of oxygen-containing functional groups on the support surface. At pH 7.4 the activity of immobilized lipase on the KAU_{OX} surface increased on 6 %, which may be due to the cumulative effect of carrier and enzyme in saturated fat hydrolysis reaction.

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ЛІПАЗОПОДІБНА АКТИВНІСТЬ НАНОПОРУВАТИХ ВУГЛЕЦЕВИХ МАТЕРІАЛІВ

Досліджено ліпазоподібну активність нанопоруватих вуглецевих матеріалів (активоване вугілля типу СКН та КАУ і їхні модифіковані форми) в реакції розкладання п-нітрофенілпальмітату за pH 6,0–8,0. Встановлено, що окиснені форми вугілля $СКН_{ок}$ та $КАУ_{ок}$ виявляють власну каталітичну активність, яка становить, відповідно, 25 % та 12 % від активності нативного ферменту. Ліпаза Candida rugose, фізично іммобілізована на поверхні нанопоруватих вуглецевих матеріалів, зберігає 30–50 % активності. Як допування вуглецевої поверхні гетероатомами, так і структурні характеристики носія впливають на активність іммобілізованого ензиму.

Ключові слова: нанопоруваті вуглецеві матеріали, ліпаза, іммобілізація ензиму, каталітичний гідроліз, п-нітрофенілпальмітат.

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ЕЛЕКТРИЧНІ ТА МЕХАНІЧНІ ВЛАСТИВОСТІ ПОЛІУРЕТАНОВИХ КОМПОЗИТІВ З ВУГЛЕЦЕВИМИ НАНОТРУБКАМИ РІЗНОЇ БУДОВИ

Досліджено електропровідність, міцність на розрив та подовження при розриві композиційних матеріалів на основі сітчастих поліуретанів з багатошаровими вуглецевими нанотрубками різної будови та геометрії залежно від концентраційного вмісту наповнювача. Встановлено, що при зростанні вмісту наповнювача спостерігається зростання електропровідності, збільшення міцності на розрив та зменшення подовження при розриві наповнених поліуретанових композитів. При цьому найвищими значеннями електропровідності та міцності на розрив в усьому концентраційному діапазоні характеризуються системи з більшим зовнішнім діаметром вуглецевих нанотрубок. Наявність атомів нітрогену у вузлах нанотрубок погіршує електропровідність та незначно впливає на міцність поліуретанових композитів.

Ключові слова: поліуретани, вуглецеві нанотрубки, електропровідність, міцність на розрив, подовження при розриві.