Catalytic and antimicrobial properties of α -amylase immobilised on the surface of metal oxide nanoparticles

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Abstract

New methods of obtaining products containing enzymes reduce the costs associated with obtaining them, increase the efficiency of processes and stabilize the created biocatalytic systems. In the study a catalytic system containing the enzyme α -amylase immobilized on ZnO nanoparticle and Fe₃O₄ nanoparticles was created. The efficiency of the processes was obtained with variables: concentrations of enzymes, temperatures and times, to define the best conditions for running the process, for which were determined equilibrium and kinetics of adsorption. The most effective parameters of α -amylase immobilization on metal oxides were determined, obtaining 100.8 mg/g sorption capacity for ZnO and 102.9 mg/g for Fe₃O₄ nanoparticles. Base on the best parameters, ZnO- α -amylase was investigated as an antimicrobial agent and Fe₃O₄- α -amylase was tested as a catalyst in the process of starch hydrolysis. As a result of the conducted experiments, it was found that α -amylase immobilized on Fe₃O₄ nanoparticles maintained high catalytic activity (the reaction rate constant K_M = 0.7799 [g/dm³] and the maximum reaction rate V_{max} = 8.660 [g/(dm³min)]).

Keywords α -amylase · Metal oxide nanoparticles · Immobilization · Nanobiocatalytic material

Introduction

Enzymes in industry, especially in biocatalysis, become increasingly popular. Enzymes are macromolecules that act as specific (bio)catalysts, accelerating or enabling chemical reactions by reducing the activation energy of the process [1, 2]. New methods of enzyme processing are aimed at reducing the costs associated with obtaining them, increasing the efficiency of processes and stabilizing the created biocatalytic systems [3]. Nanotechnological methods are becoming widely used to overcome these problems. The increased surface area of the nanoparticles makes the immobilization of enzymes effective, which ultimately promotes the catalytic activity of proteins [4, 5].

An α -amylase classified as EC 3.2.1.1 belongs to glycoside hydrolases and acts on glycosidic bonds, exactly on α -1,4-glycosidic bonds [6]. The enzyme decomposes starch

to two or three saccharides. In the human body it occurs in saliva and is secreted by the pancreas. The amylase group is used in industries related to fermentation processes or starch decomposition [7, 8].

An immobilisation of enzymes is intended to improve the resistance of biocatalysts to changing reaction conditions that adversely affect the catalytic performance of the free enzyme. The sensitivity of enzymes to changes in process conditions results from their composition and a change in the structure of enzymes may cause their complete inactivation [9]. Immobilization has a positive effect on the structure of the catalytic protein by stabilizing, resulting in increased tolerance to pH, temperature or denaturants. Modification of the protein by connecting it to the carrier facilitates its separation from the reaction mixture after the process. The resulting products are not contaminated with the enzyme and the enzyme deposited on the carrier can be reused. The immobilization of the biocatalyst also extends its catalytic activity so that it can be used repeatedly [10]. However, the immobilisation process may be associated with the deterioration of the catalytic properties of the entire biocatalyst. This is due to the stiffening of the protein structure and the restriction of the

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transport of substrates and products to and from the place of the active enzyme [11].

Enzymes are characterized by additional catalytic functions. In addition to their catalytic functions, some enzymes present additional properties, e.g. antimicrobial. Depending on the future use of the enzymes, as carriers of enzymes ZnO, Fe₃O₄, SiO₂, TiO₂, Ag or Au nanoparticles can be commonly used [12, 13]. By using enzymes combined with selected nanomaterials that show biocidal activity (CuO, ZnO, Ag, Cu), it is possible to obtain products with synergistic effect [14, 15]. The antimicrobial properties are distinguished by enzymes from the hydrolysis group (e.g. glycosidase and peptidase) due to the possibility of decomposition of peptidoglycans contained in cell walls, as well as oxidoreductase (e.g. oxidase), which antimicrobial activity is indirect and results from products catalysed by them [16, 17].

The immobilisation by an adsorption on the surface of insoluble carrier is a method often appropriate. The method is based on the formation of bonds between the enzyme and the carrier resulting from hydrogen, hydrophobic, electrostatic, ionic and van der Waals forces [18]. The weak interactions do not influence the tertiary structure of the enzyme, which allows maintaining high catalytic activity. At the other side, the bonds can easily be broken off, so that the protein can be washed away, reducing the activity of the preparation. Through its ease of application of immobilization via adsorption and the low cost of the method, it is a widely used technique. The adsorption enables the use of many types of media that are selected for a sufficiently high protein affinity to the matrix. Depending on the choice of carrier, as well as the enzyme used and the parameters of the process environment, the amount of immobilized enzyme changes [19]. The covalent immobilisation is a method that involves the formation of stable and permanent chemical bonds between the enzyme and the carrier, which limits the leaching of the enzyme from the matrix, extending the activity of the product [20].

The aim of the study was to carry out the process of α -amylase immobilization on the surface of metal oxide nanoparticles and to obtain materials with antimicrobial and biocatalytic properties. The scope of the study included the determination of the influence of the parameters of the process of obtaining materials on the efficiency of this process, analysis of equilibrium and kinetic parameters of the process and determination of the activity of α -amylase immobilized on Fe₃O₄ in the process of hydrolysis of starch and antimicrobial properties of α -amylase immobilized on ZnO nanoparticles. The Fe₃O₄ nanoparticles allow to separate the biocatalyst from the reaction system, while ZnO nanoparticles allow to obtain material with the increased antimicrobial activity.

Materials and methods

Materials

Iron(III) chloride $FeCl_3 \cdot 6H_2O$ (Sigma Aldrich) and iron(II) sulphate $FeSO_4 \cdot 7H_2O$ (Sigma Aldrich) were used as precursors of iron oxide nanoparticles. Sodium hydroxide (NaOH Sigma Aldrich) and sodium carbonate (Na_2CO_3 (Sigma Aldrich)) were used as a precipitating agent. The enzyme tested was α -amylase from *Aspergillus oryzae* (Sigma Aldrich).

Synthesis of Fe₃O₄ and ZnO nanoparticles

The solutions of $FeCl_3 \cdot 6H_2O$ and $FeSO_4 \cdot 7H_2O$ were added to the Teflon vessel in the molar ratio of iron ions 1:1. In the presence of ultrasounds (Hielscher UP400St sonicator), an aqueous solution of NaOH acting as a precipitating agent of iron hydroxide was injected and the mixture was homogenised for 3 min. Then, in the MAGNUM II microwave reactor at 180 °C for 15 min, a dehydration process was carried out to obtain Fe₃O₄ nanoparticles. The zinc oxide nanoparticles were obtained in the presence of ultrasound by dropping into a solution of $ZnSO_4 \cdot 7H_2O$ a solution of Na_2CO_3 (for complete precipitation of zinc hydroxide) and after 3 min the suspension was in the microwave reactor at 180 °C for 10 min. The materials were filtered, washed and dried at 105 °C for 24 h. The schematic diagram for the process was presented in Fig. 1.

Preparation of α -amylase immobilised on metal oxide nanoparticles

To the solution of α -amylase from *Aspergillus oryzae* (Sigma Aldrich) with variable initial enzyme concentration 100 mg of Fe₃O₄ or ZnO nanoparticles were added. The process was carried out at 20–30 °C in the time from 30 to 120 min. At the end of the process, the solution was filtered and the filtrate was examined spectrophotometrically to analyse the enzyme concentration and assess the efficiency of the process. The process efficiency (E) was determined from the equation:

$$E = \frac{C_{enzyme,0} - C_{enzyme,t}}{C_{enzyme,0}} \cdot 100\%$$

where: $C_{enzyme,0}$ —spectrophotometrically measured concentration of the α -amylase solution [mg/dm³], $C_{enzyme,t}$ —spectrophotometrically measured concentration of the α -amylase after sorption process [mg/dm³]. The amount of material that was adsorbed in the ratio of the amount of oxide (q) was determined from the equation:

Fig. 1 Schematic diagram of the process

$$q = \frac{(C_{enzyme,0} - C_{enzyme,t}) \cdot V \left[\frac{mg}{g} \right]}{m_{MeO_x}}$$

where: m_{MeO_x} —mass of metal oxide nanoparticles [mg],
 V —volume of the enzyme solution.

Table 1 contains the variables tested in the process of the immobilisation of α -amylase on the metal oxides nanoparticles with ranges of variability of input quantities.

On the basis of statistical analysis, the variables and the way to which they affected the efficiency of the sorption process were determined. The analysis was carried out using the program STATISTICA 10.0. By determining

Table 1 Range of variability of input quantities of the α -amylase immobilisation process on metal oxide nanoparticles

Input variable	Symbol	Unit	Minimum value	Maximum value
Temperature	T	[°C]	20	30
Initial enzyme concentration	C_{enz}	[g/dm ³]	0,1	1
Sorption time	t	[min]	30	120

these parameters, the most favourable conditions for the immobilisation process were selected. Then, the sorption was carried out at selected parameters in order to obtain the most suitable system of the metal oxide nanoparticles with the enzyme for further research.

Kinetic and equilibrium parameters of the α -amylase immobilisation process

The equilibrium and the kinetics of the processes were examined. Analogously to the study of immobilization efficiency, 100 mg of the metal oxides were prepared and then 10 cm³ of enzyme solution of proper concentration was added. After the process, the solution was examined spectrophotometrically. The equilibrium parameters and

process kinetics were determined using selected models (Table 2).

Material characteristics

The UV–Vis spectroscopy was used to quantify the α -amylase content in the solutions. The determination method was based on modified Lowry's method [23]. The measurement was performed at 670 nm with the Rayleigh UV-1800 spectrophotometer.

The prepared mixtures: A: 2% solution of Na₂CO₃ in 0.1 M NaOH, B: 1% solution of CuSO₄. C: 2% sodium tartrate solution, D: 100 cm³ of solution A + 1 cm³ B + 1 cm³ C, prepared 30 min before the measurement, E: Folin-Ciocalteu's solution diluted with distilled water in the ratio

Table 2 Examined equilibrium models and sorption kinetics [21, 22]

Equilibrium models sorption processes			
Isotherms	Non-linear form	Linear form	Descriptions
Langmuir	$q = \frac{q_{max} \cdot K_L \cdot C}{1 + K_L \cdot C}$	$\frac{1}{q} = \frac{1}{C} \cdot \frac{1}{q_{max} \cdot K_L} + \frac{1}{q_{max}}$	q_e —equilibrium sorption capacity [mg/g],
Freundlich	$q = K_F C^{\frac{1}{n}}$	$\log(q) = \log(K_F) + \frac{1}{n} \log(C)$	C_e —equilibrium concentration of adsorbate [mg/dm ³],
Temkin	$q = \frac{RT}{b_T} \ln(A_T C)$	$q = \frac{RT}{b_T} \ln(A_T) + \frac{(RT)}{b_T} \ln(C)$	q_m —maximum sorption capacity [mg/g], K_L —Langmuir's constant [mg/g], n —Freundlich's constant on sorption intensity, K_F —Freundlich's adsorption capacity constant, K_T —Temkin's constant [dm ³ /g], b_T —Temkin's isotherm constant, R —universal gas constant [kJ/(mol•K)], T —temperature [K];
Kinetics models sorption processes			
Model	Non-linear form	Linear form	Descriptions
Pseudo-first order	$\frac{dq_t}{dt} = k_I (q_{max} - q_t)$	$\log(q_{max} - q_t) = \log(q_{max}) - \frac{k_I}{2,303} t$	q_t —sorption capacity after time t [mg/g],
Pseudo-second order	$\frac{dq_t}{dt} = k_{II} (q - q_t)^2$	$\frac{t}{q_t} = \frac{1}{k_{II} q^2} + \frac{1}{q} t$	q_e —equilibrium sorption capacity [mg/g],
Elovich	$\frac{dq_t}{dt} = \alpha \exp(-\beta q_t)$	$q_t = \frac{1}{\beta} \ln(\alpha \beta) + \frac{1}{\beta} \ln(t)$	t —time [min],
Intramolecular diffusion	$R = k_{id}(t)^a$	$\log(R) = \log(k_{id}) + a \log(t)$	k_I —pseudo-first order constant [min ⁻¹], k_2 —pseudo-second order constant $\left[\frac{g \cdot dm^3}{mg \cdot min} \right]$, α —initial rate of adsorption $\left[\frac{mg}{g \cdot min} \right]$, β —adsorption constant, R —share of absorbed particles [%], k_{id} —constant rate [min ⁻¹], a —constant depending on the system

1:1. The samples for spectrophotometric tests were prepared by adding 1 cm³ of the test solution, 5 cm³ of solution D, after 10 min 0.5 cm³ of reagent E was added and the sample remained in the dark. After 30 min, the absorbance of the samples was measured at 670 nm. The reference curves were made using α -amylase.

Studies on crystalline structure of metal oxides were carried out using Philips X'Pert Camera diffractometer with PW 1752/00 CuK α monochromator in the range of angles 2 θ , from 10 to 60°. The size of nanoparticles crystallites based on the metal oxides was determined on the basis of Scherrer's equation:

$$d = \frac{K \cdot \lambda}{FWHM \cdot \cos \theta}$$

where: d—average size of crystallites, FWHM—peak width at half of its height, which is proportional to the size of the crystallite, K—Scherrer constant, depending on the shape of the particles (for cuboid-shaped particles it takes the value of 0.89; 1.10 for spherical particles), λ —X-ray wavelength, θ —angle which is formed by the incident radiation with the atomic plane. The constant K was selected on the basis of the shape of particles determined by SEM and TEM microphotography.

The morphology of the obtained metal oxide nanoparticles was examined using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The investigations were carried out using VegaII-Tescan Company and Tecnai Transmission Electron Microscope, F20 X-Twin. The Fourier Transform Infrared Spectroscopy (FT-IR) was used to determine the presence of specific functional groups in the systems studied. The FT-IR measurements were carried out using Nicolet 380 apparatus in which spectra were recorded in the range from 3900 to 400 cm⁻¹. The sorbent surface and diameter of pores were performed (Micromeritics ASAP2010). In order to determine the energy gap width of metal oxide nanoparticles, diffuse reflectance spectroscopy was measured in the range 200–850 nm. Reflective spectra were recorded with a UV-2600 spectrophotometer (Shimadzu) equipped with an integrating sphere of 10 cm diameter. The nature of the surface of the metal oxide nanoparticles was determined on the basis of the zeta electrokinetic potential (Malvern Instruments, ZS-90 and Brookhaven, zetaPALS).

Studies on the biocatalytic properties of α -amylase for Fe₃O₄

The aim of the study was to check the activity of the immobilized enzyme and its applicability as a bio-nanocatalytic material. The starch hydrolysis process was carried out using the obtained immobilized enzyme on the iron oxide nanoparticles. The starch solution from 1 to

10 g/dm³ was added to the iron oxide with α -amylase and then starch distribution during time was studied. Using the magnetic properties of the carrier, the enzyme was separated from the reaction mixture. At the next stage, 10 ml of post-reaction mixture solution was measured into a beaker, water and 0.1 ml of 0.01 M I₂ solution in 1% KI were added. The solution was mixed on a magnetic stirrer. After 10 min the absorbance of the solution was measured on a spectrophotometer.

The kinetic parameters of this process were determined i.e. Michaelis' constant and the maximum reaction velocity was calculated from the Lineweaver–Burk equation [18]:

$$\frac{1}{V} = \frac{K_M}{V_{\max}} \cdot \frac{1}{S} + \frac{1}{V_{\max}}$$

where: V—reaction rate [g/(dm³min)], K_M—constant Michaelis–Menten [g/dm³], V_{max}—maximum reaction rate [g/(dm³min)], S—substrate concentration [g/dm³].

Studies on antimicrobial properties of α -amylase for ZnO

The antimicrobial properties were studied by observing the growth of selected microorganisms from the bacterial group. The examined microorganisms were *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. The test substances were: zinc oxide, zinc oxide with α -amylase and α -amylase. The weights containing the oxide constituted a specific percentage of the nutrient solution weight (about 15 g)—0.1%, 0.5% and 1%. The size of the weights containing the enzymes themselves was calculated taking into account the efficiency of the sorption process, so that the content of the enzyme is equal to the content of the enzyme immobilized on zinc oxide. On the dishes with prepared appropriate mediums, the weights of the tested substances were applied and then the cultures were made by surface method. Growth was observed after 24 h, 48 h and 72 h after the cultures. The tested samples with microorganisms were incubated at 30 °C. The effect of materials on microorganisms was evaluated according to the given scale (Table 3).

Results

Physicochemical characteristics of α -amylases on the metal oxide nanoparticles

The phase composition of the obtained ZnO and Fe₃O₄ oxides was examined using the XRD method (Fig. 2). Based on the obtained diffractogram and the Scherrer equation the average size of nanoparticle crystallites was determined using. The average size of crystallites in the

Table 3 The assessment of bacterial and fungal growth in the presence of tested materials

Growth intensity	Assessment
0	No visible growth under the microscope
1	Growth invisible to the naked eye but clearly visible under a microscope
2	Growth visible to the unarmied eye, covering up to 25% of the test area
3	Growth visible to the unarmied eye, covering up to 50% of the test area
4	Significant increase, covering more than 50% of the test area
5	Intense growth, covering the entire test area

Fig. 2 Diffractogram of ZnO and Fe₃O₄ nanoparticles synthesised in a microwave reactor

obtained zinc oxide was 81.18 nm and for the iron oxide was 23.24 nm.

The morphology of the pure metal oxide nanoparticles was studied using scanning electron microscopy and transmission electron microscopy. In Fig. 3a–f it can be observed that the zinc oxide obtained is mostly needle-shaped particles. The zinc oxide nanoparticles form elongated agglomerates up to about 10 μm in size. Single needles of zinc oxide are about 100 nm long and 5 nm thick. The nanoparticles of the iron oxide are spherical (Fig. 3g–l). The particle diameter is about 20 nm. Nanoparticles do not occur in the form of agglomerates; single particles are clearly visible. The dimensions of metal oxide nanoparticles are consistent with the size of crystallites determined by XRD method.

The BET surface area of metal oxide nanoparticles was measured as 10.92 m²/g and 16.67 m²/g, respectively, for Fe₃O₄ and ZnO nanoparticles (Table 4). The study revealed that the adsorption of enzyme molecules took place mainly on the surface of oxides, not in their pores. The small size of nanoparticles increased the effectiveness of contact with α-amylase, without increasing the porosity of the material, so that the availability of α-amylase was maintained. According to Pandya et al. if the enzyme is immobilized on the surface of mesoporous silica, it is immobilized only on the external surface, which results in increased activity

with limited stability of the whole system. For materials with a larger pore diameter, the enzyme is immobilized inside the pores and a high increase in enzyme stability is observed [24]. An alternative method of improving system stability was given by Patel et al., who modified the surface of Fe₃O₄ nanoparticles with reduced graphene oxide. Additionally, the use of graphene oxide improved the catalytic properties of the enzyme, the activity of which increased by 192% compared to the free enzyme [25].

The ZnO and Fe₃O₄ nanoparticles were characterized by a positively charged surface with an electrokinetic potential of approximately 20 mV. Go et al. confirmed that the hydrodynamic radiation and zeta potentials of NPs were highly affected by sorbed compounds. The mean zeta potential of ZnO NPs in their study was determined to be 22.9 ± 1.1 mV. A positively charged surface increases the binding efficiency of -COO- and -NH₂ groups derived from α-amylase [26].

The samples of zinc oxide, zinc oxide with immobilized α-amylase, iron oxide and iron oxide with immobilized α-amylase were examined by infrared spectroscopy (Fig. 4). The band associated with the bond Zn–O is found at a wavelength number of approximately 465 cm⁻¹. The band associated with the bond O–H present in the material has been confirmed at a wavelength number of approximately 3382 cm⁻¹ [27, 28]. On the spectrum obtained for zinc oxide with immobilized α-amylase, a peak with a wavelength of 1652 cm⁻¹ is observed, it is one of the characteristic peaks appearing in the diagrams of α-amylase and is associated with oscillations of N–H bonds [29, 30]. For pure iron oxide, in the area 500–600 cm⁻¹, bands derived from the Fe–O bond in nanoparticles can be observed, while in the area 3400–3500 cm⁻¹ the band is associated with the presence of hydroxyl groups derived from O–H bonds. The FT-IR analysis showed the presence of α-amylase immobilized on iron oxide. After adsorption of α-amylase a new band within 1400–1500 cm⁻¹ appeared, which may indicate amide bond.

Fig. 3 The SEM microphotographs of ZnO, extension: 3A-1 000x, 3B-5 000x, 3C-200 000x; the TEM microphotographs of ZnO, extension: 3D-36 000x, 3E-150 000x, 3F- 750 000x; the SEM

microphotographs of Fe_3O_4 , extension: 3G-1 000x, 2H-5 000x, 3I-200 000x; the TEM microphotographs of Fe_3O_4 , extension: 3J-36 000x, 3K-150 000x, 3L-750 000x;

Table 4 Surface properties of metal oxide nanoparticles

	ZnO NPs	ZnO- α -amylase	Fe ₃ O ₄ NPs	Fe ₃ O ₄ - α -amylase
BET Surface [m ² /g]	16.67	–	10.92	–
Pore volume [cm ³ /g]	0.07707	–	0.06172	–
Pore size [nm]	2.76	–	22.61	–
Energy gap width	3,54	3,17	1,96	1,94
Electrokinetic potential ξ [mV]	23.1	22.5	18.6	18.2

Fig. 4 Spectra of metal oxide nanoparticles and α -amylase immobilized on the surface of metal oxide nanoparticles

Immobilisation of α -amylase on metal oxide nanoparticles

Table 5 presents averaged results of the α -amylase immobilization process. Figure 5 a,b presents an analysis of the effect of the examined parameters on the efficiency of the process (E) of α -amylase immobilization on the zinc oxide. The initial concentration of the enzyme and temperature had the greatest influence on the efficiency of enzyme deposition. The efficiency of enzyme immobilization was significantly influenced by time (linearly) and interactions between temperature and time and between enzyme concentration and temperature. In the processes of obtaining α -amylase for Fe₃O₄ the significance of the effect was confirmed for the initial enzyme concentration.

Table 5 Sorption efficiency results for α -amylase to ZnO and α -amylase to Fe₃O₄

L.p	C_{enz} [g/dm ³]	T [°C]	t [min]	ZnO- α -amylase		Fe ₃ O ₄ - α -amylase	
				Sorption efficiency E [%]	Sorption capacity [mg/g]	Sorption efficiency E [%]	Sorption capacity [mg/g]
1	0.1	20	30	≥ 99.99	6.27 ± 0.3	92.81 ± 12.46	6.27 ± 0.33
2	0.1	20	120	≥ 99.99	6.66 ± 0.0	99.94 ± 0.11	7.99 ± 0.81
3	0.1	25	75	≥ 99.99	6.68 ± 0.0	99.91 ± 0.15	6.37 ± 0.54
4	0.1	30	30	≥ 99.99	6.27 ± 0.4	97.03 ± 5.14	7.15 ± 0.70
5	0.1	30	120	≥ 99.99	6.68 ± 0.0	78.93 ± 12.14	85.46 ± 22.32
6	0.5	20	75	94.68 ± 0.76	50.37 ± 0.7	86.78 ± 1.07	102.9 ± 12.26
7	0.5	25	30	95.96 ± 2.53	51.18 ± 2.3	83.12 ± 1.66	83.13 ± 3.28
8	0.5	25	75	97.68 ± 2.09	51.98 ± 2.0	85.20 ± 2.22	89.75 ± 3.19
9	0.5	25	75	96.92 ± 2.91	51.49 ± 2.5	99.99 ± 0.01	7.75 ± 3.28
10	0.5	25	120	≥ 99.99	54.17 ± 0.2	88.22 ± 6.05	86.63 ± 15.10
11	0.5	30	75	97.26 ± 3.25	46.22 ± 4.1	94.05 ± 9.24	47.10 ± 4.11
12	1.0	20	30	86.35 ± 1.38	85.56 ± 0.8	85.96 ± 1.49	44.48 ± 2.40
13	1.0	20	120	96.20 ± 2.81	100.8 ± 8.2	90.49 ± 1.14	46.12 ± 1.52
14	1.0	25	75	93.79 ± 2.05	97.31 ± 7.4	92.84 ± 6.28	48.35 ± 5.45
15	1.0	30	30	98.77 ± 0.54	98.12 ± 0.7	91.94 ± 10.60	48.03 ± 7.35
16	1.0	30	120	94.77 ± 4.57	99.44 ± 12.1	93.26 ± 6.24	48.58 ± 5.23

Fig. 5 Parameters significantly influencing the efficiency of the process (E) of α -amylase immobilization: **a**, **b** immobilization of α -amylase on ZnO (significance level equal to 5%) and approximation

profile of predicted values; **c**, **d** immobilization of α -amylase on Fe₃O₄ and approximation profile of predicted values

It was found that the most optimal process parameters are: enzyme concentration equal to 1 g/dm³, temperature 30 °C and time of 120 min. The conditions ensure optimal immobilization efficiency, with the maximum amount of immobilized enzyme. The amount of absorbed protein increases significantly with the concentration, despite the reduced efficiency of sorption. A higher process temperature increases the efficiency of the process without significantly affecting the amount of absorbed enzyme. On the basis of the results, sorption of the enzyme to Fe₃O₄ was carried out in the most favourable conditions, i.e. temperature at 20 °C for 120 min in the enzyme solution of 1 g/dm³ concentration.

Kinetic and equilibrium parameters of the α -amylase immobilisation process

Based on the results, the parameters of sorption equilibrium of the enzyme on the surface of the metal oxides were determined (Table 6). For both oxides, Langmuir isotherm has the highest coefficients of determination. The Langmuir isotherm describes the chemical adsorption which results in a single-molecular layer of the sorbent [22]. The high determination factor for Freundlich's isotherm, which concerns multilayer physical adsorption on microporous materials has also been confirmed for α -amylase deposition on ZnO [21]. It can be concluded that the sorption process of α -amylase on zinc oxide is a complex process, following the assumptions of these two models (Fig. 6). The analysis of the kinetics of enzyme immobilization processes confirmed the chemical nature of the processes. The sorption

Table 6 The equilibrium and the kinetics parameters of the α -amylase immobilisation process on the metal oxides nanoparticles

Equilibrium models sorption processes		ZnO- α -amylase	Fe ₃ O ₄ - α -amylase	Kinetics models sorption processes		ZnO- α -amylase	Fe ₃ O ₄ - α -amylase
Langmuir	q_{max}	427.8	276.2	Pseudo-first order	k_f	0.015203	0.004907
	K_L	0.2936	0.4534		q_{max}	47.5335	25.6109
	R^2	0.9992	0.9993		R^2	0.9505	0.7261
Freundlich	K_F	98.99	92.27	Pseudo-second order	k_{II}	2.1606	2.3222
	n	1.1381	0.9399		q_{max}	101.53	103.87
	R^2	0.9991	0.9882		R^2	0.9903	0.9746
Temkin	A_T	6.3396	9.4548	Elovich	α	0.9617	6.99
	b_T	46.2778	64.5844		β	20.87	91.743
	R^2	0.9726	0.9139		R^2	0.9364	0.7211
				Intramolecular diffusion	k_{id}	0.4583	0.5226
					a	0.2181	0.1196
					R^2	0.9404	0.7851

Fig. 6 Plots of α -amylase isothermal equilibrium sorption on metal oxide nanoparticles: **a** sorption of α -amylase on ZnO nanoparticles, **b** sorption of α -amylase on Fe₃O₄ nanoparticles

processes of α -amylase to ZnO and Fe₃O₄ were carried out according to the kinetics of the pseudo-second order, which additionally confirms the advantage of chemical nature of the enzyme sorption. On the basis of the literature it was found that mainly carboxylic and amino groups take part in the binding of enzymes to the surface of oxides. The presence of polar groups such as carboxylic (–COOH), phosphate (–PO(OH)₂), amino (–NH₂), and thiol (–SH) groups anchor the molecule on the surface of metal oxides such as ZnO. The ZnO surface is characterized by the polar part resulting from the presence of oxygen and the neutral part associated with the presence of Zn. The carboxylic groups present in amylase bind to the oxide through dissociative adsorption through dissociated hydrogen atom with surface oxygen, while amine and hydroxyl groups tend to bind non-associative [31, 32]. Kahraman et al. confirmed the α -amylase bonding by the covalent bonds immobilized on glass beads containing phthalic chloride. The functioning of the carrier surface with amine groups

caused prolongation of the enzyme activity. Higher thermostability compared to free enzyme particles was also confirmed [33]. Pandya et al. observed the significance of the α -amylase deposition site for its activity [24].

The catalytic properties α -amylases on Fe₃O₄

The catalytic properties of α -amylases were tested by hydrolysis of starch. The plot of starch decomposition over time is presented in Fig. 7a. The kinetic parameters of the reaction were determined after the hydrolysis of starch. The concentration of hydrolysis product (S) was calculated, based on the concentration of starch remaining in the system. Next, a diagram 1/S from 1/t was plotted, which is presented in Fig. 7b. This graph represents the Lineweaver–Burk equation. On the basis of the function equation the constant of K_M reaction rate = 0.7799 [g/dm³] and the maximum reaction rate V_{max} = 8.660 [g/(dm³min)] were calculated. Despite the limited mobility of the

Fig. 7 **a** The starch concentration during hydrolysis in the presence of the Fe_3O_4 - α -amylase, **b** The rate of starch hydrolysis in the presence of the Fe_3O_4 - α -amylase

enzyme and contact with the reagent, the immobilization of α -amylase on the carrier surface increases the reaction area and preserves the efficiency of the catalytic process.

The effectiveness of α -amylase combined with the carrier was confirmed by Salaonkar et al. comparing the effectiveness of hydrolysis distribution. The constant reaction rate for the bound enzyme was $0.5889 \mu\text{mol}/\text{dm}^3$ which is consistent with the results obtained in the presented study. Compared to the reaction carried out in the presence of a free enzyme, the value of both the constant and the maximum reaction rate was comparable, which confirmed the effectiveness of deposition of the enzyme on a carrier without loss of its activity [34].

Antimicrobial properties

The antimicrobial properties of ZnO - α -amylase, pure zinc oxide and pure enzyme as reference samples were tested. Table 7 summarises the data obtained in the study. The antimicrobial activity of zinc oxide nanoparticles and α -amylase on ZnO nanoparticles surfaces was confirmed. The zinc oxide showed antimicrobial properties against bacteria both gram positive and gram negative. The fungicidal properties of nanoparticles were limited. Azizi-Lalabadi et al. proved that the antimicrobial activity of ZnO nanoparticles is dependent on the species of microorganism (gram positive or gram negative). The sensitive microorganisms were gram negative bacteria, i.e. *P. fluorescens*, and *E. coli* [35].

Both Zn^{2+} particles and ZnO nanoparticles have an antibacterial effect. The nanoparticles reveal biocidal activity for two mechanisms: as a result of free metal ion toxicity resulting from dissolution of metals from the surface of the nanoparticles and generation of oxidative stress through generation of reactive oxygen species (ROS) using hydrogen peroxide (H_2O_2) on the surface of the nanoparticles. Additionally, nanoparticles can affect the viability of microorganisms by agglomeration on the surface of

bacteria and change the structure of lipids, peptidoglycans, proteins and their DNA [36].

In the presented studies, α -amylase itself demonstrated absence of antimicrobial activity. However, the addition of α -amylase improved the bioactivity of ZnO NPs. The enzyme catalyses the hydrolysis of the internal α -1,4-glycosidic bonds in starch, transforming starch into low molecular weight products such as glucose, maltose and maltotriose compounds. The basic components of the cell membrane are lipids and proteins which together make up 60 to 80%. The remaining part is made up of sugars, which are bound to lipids and proteins forming glycolipids and glycoproteins. The α -amylase affects the decomposition of sugars; however, it is only when combined with zinc oxide nanoparticles that the cell membrane of microorganisms is destroyed, thus deactivating them. Therefore, it is beneficial to obtain a complex compound with synergistic properties. Date described the process of glycoprotein breakdown using α -amylase confirming its effectiveness [37].

The activity of the composite results from the effectiveness of the connection between the components. Excessive release of α -amylase would reduce contact between ZnO and the enzyme and the activity of such a system would be equal to the pure ZnO . Anthony et al. confirmed the synergistic effects of amylase with nanoparticles on the example of silver nanoparticles. Despite the confirmed antimicrobial properties of Ag NPs, the connection of the material with the enzyme additionally increases the activity of silver nanoparticles [38]. Cui et al. by modifying the surface of Fe_3O_4 with silica received a material with an inactive surface, which protected the catalase from biological, thermal and chemical degradation. However, the inactive surface inhibited activity of the carrier [39]. Homaei and Saberi successfully immobilized α -amylase on gold nanoparticles while maintaining temperature stability compared to free α -amylase. The enzyme also showed higher activity than the free form. Due to the improved stability of the enzyme on the surface of gold

Table 7 The antimicrobial properties of ZnO, ZnO- α -amylase and α -amylase for *E. coli*, *S. aureus* and *C. albicans*

C_p [%]	<i>E. coli</i>						<i>S. aureus</i>						<i>C. albicans</i>					
	ZnO						ZnO						ZnO					
	0.1%		0.5%		1.0%		0.1%		0.5%		1.0%		0.1%		0.5%		1.0%	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
t [h]																		
24	2	2	2	1	0	0	0	1	0	1	0	0	3	3	3	3	3	3
48	2	2	2	1	0	0	2	2	2	3	2	2	3	3	4	4	4	4
72	2	2	2	1	0	0	3	3	2	3	3	2	4	4	4	4	4	4

C_p [%]	ZnO + α -amylase						ZnO + α -amylase						ZnO + α -amylase					
	0.1%		0.5%		1.0%		0.1%		0.5%		1.0%		0.1%		0.5%		1.0%	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
24	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	3	3	3
48	1	1	1	1	0	0	1	2	1	1	1	1	3	3	4	3	4	3
72	1	2	2	1	0	0	3	3	2	2	2	2	4	4	4	4	4	4

C_p [%]	α -Amylase						α -Amylase						α -Amylase					
	0.1%		0.5%		1.0%		0.1%		0.5%		1.0%		0.1%		0.5%		1.0%	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
24	2	2	2	2	2	2	3	3	3	3	3	3	3	3	3	3	3	3
48	2	2	2	2	2	2	3	3	4	3	4	3	4	4	4	4	4	4
72	2	2	2	2	2	2	4	4	4	4	4	4	4	4	4	4	4	4

nanoparticles, the authors confirmed the possibility of using the material in industry as a biocatalyst for the production of glucose syrups on an industrial scale [13].

Conclusion

In the processes of α -amylase immobilization on the surface of metal oxide nanoparticles ZnO- α -amylase and Fe_3O_4 - α -amylase materials were obtained.

In the processes of immobilization of the enzyme on the ZnO surface, the initial concentration of the enzyme, process time and its temperature showed to be important parameters. The most effective parameters of α -amylase immobilization on zinc oxide were determined, obtaining 96.20% efficiency of the enzyme sorption process and 100.8 mg/g carrier. The process of enzyme immobilization is complex—there is chemical adsorption and multilayer physical adsorption. Antimicrobial properties of prepared amylase from ZnO result from synergic action of zinc oxide nanoparticles with the enzyme, and α -amylase itself did not show antimicrobial properties.

In Fe_3O_4 - α -amylase systems the obtained iron oxide particles had magnetic properties. By using iron oxide

nanoparticles with magnetic properties, it is possible to separate the biocatalyst from the reaction system. The most favourable parameters for sorption of α -amylase to Fe_3O_4 were: temperature 20 °C, concentration of enzyme solution 1 g/dm³, sorption time 120 min. In the studies on the deposition of the enzyme on the surface of metal oxide nanoparticles, the equilibrium and kinetics parameters of the process were determined, based on which the chemical character of the enzyme immobilization was found. The model of pseudo-second order kinetics was characterized by the best fit (R^2) for Fe_3O_4 - α -amylase. This indicated that chemisorption was taking place. The Langmuir isotherm showed the best fit for the study. The parameters of starch hydrolysis process indicated the catalytic activity of the enzyme immobilized on Fe_3O_4 nanoparticles.

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Compliance with ethical standards

Conflict of interest The authors report no declarations of interest.

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