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Oxidoreductase Immobilization on Magnetic Nanoparticles

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The aim of this work is to develop the synthesis of the magnetically separable biocatalyst for enzymatic oxidation of D-glucose to D-gluconic acid with high product yields. The biocatalyst support is based on magnetite particles (MPs) synthesized by coprecipitation and coated with the amino- silica layer to facilitate further functionalization. This functionalization involves the attachment of the glutaric dialdehyde linker followed by the covalent attachment of glucose oxidase (GOx, an enzyme of oxidoreductase group) via its amino groups. TEM, XRD, and magnetic measurements were performed for initial MPs and GOx biocatalyst. The biocatalyst activity was studied in the oxidation reaction of D-glucose to D-gluconic acid. The biocatalyst synthesis proposed in this work allowed to create a highly effective system. It was found that GOx immobilized on the modified MPs was the most active biocatalytic system in comparison with other inorganic support and showing 88% of gluconic acid yield.

1. Introduction

The reactions of monosaccharides oxidation are of great importance in the synthesis of vitamins. A product of the oxidation of D-glucose is D-gluconic acid, which is widely used in a wide range of industries and in the household (Parimal et al*.,* 2016). D-glucose due to its polyfunctionality is oxidized with the formation of a large number of side-products. Selective oxidation of semiacetal group (without oxidation of other hydroxyl groups) demands new catalytic systems and flow technology. Nowadays chemical and electrochemical oxidation of D-glucose in the presence of different oxidants is used in gluconic acid production, but the selectivity of the process and the target product yield are generally low because of the formation of side products. Also, special implementation from anticorrosion inert materials and the use of a large number of oxidants which have an adverse environmental impact are required (Garcia-Morale et al., 2015).
Heterogeneous catalytic method for monosaccharides oxidation is quite prospective in comparison with others. A considerable quantity of works is devoted to catalytic D-glucose oxidation using mono- and bimetallic catalysts on the base of Pt (Da Via et al., 2016), Pd (Megias-Sayago et al*.,* 2017), Bi (Lee et al ., 2016), Au (Morawa Eblagon et al*.,* 2016), Co (Taketoshi et al., 2014). This method requires the development of active, stable and selective catalytic systems. In spite of numerous research, the problem of selectivity is still an open issue nowadays. Moreover the separation, even in the case of heterogeneous catalysts, can be incomplete and small particles of heterogeneous catalysts remain in the final product that requires the additional purification stage. From this point of view, magnetically separable catalysts are more perspective.
Microbiological method of monosaccharide oxidation allows using milder conditions and obtaining higher selectivity. For these purposes, different microbial strains (such as *Gluconobacter spp*., *Aspergillus niger*, *Tricholoma robustum* and *Tricholoma bakamatsutake*, *Gluconobacter oxydans,* and others) (Jagdish and Neelam, 2013) and microbial-derived enzymes are used (Caixia et al., 2016). At present enzymatic methods of gluconic acid production are widely studied, however, as the enzyme is expensive and cannot be reused it is necessary to develop new immobilized enzymatic catalytic systems – biocatalysts. The synthesis of biocatalyst based on the immobilized enzymes is the next step in the development of prospective catalytic

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systems. The use of enzymes as the catalysts in fine organic synthesis has numerous advantages: (1) mild reaction conditions in terms of temperature, pressure, pH and low energy demand; (2) process is characterized by high enantio-, regio- and chemoselectivity. The advantages of immobilized enzymes in comparison with their native form are enzyme stability increase, re-use or long-term use, reaction rate control, easy separation from the reaction products, the possibility to stop the reaction at any time, enzyme catalytic properties modulation, prevention of the reaction products contamination. Obviously, enzymes immobilization can directly affect the decrease in the process cost and the quality of the target products. Moreover, the potential change of enzyme properties through immobilization hold promise. For example, by choosing the immobilization method the temperature range of enzyme activity can be improved so that in some cases the reaction can be conducted at higher temperatures thereby increasing the reaction rate and product yield (Dwevedi, 2016).

Glucose oxidase (GOx, an enzyme of oxidoreductase group EC 1.1.3.4) is known to be used in D-glucose selective oxidation to gluconic acid (Huan et al., 2016). Authors proposed glucose oxidase immobilization on carbon nanotubes (MacAodha et al., 2013) and silica (Kumar and Leech, 2014). Nowadays biocatalysts are widely studied to estimate the influence of (I) nature of support, modifier and crosslinking agent; (II) introduction of the second enzyme; (III) conditions of enzymes immobilization and oxidation reaction with them on the catalytic process.

In the past decade, a significant growth of researchers' interest in magnetic nanoparticles (MNPs) (Kow et al., 2017) and their derived materials (Maharramov et al., 2017) is observed. The main field of magnetic materials application which has recently been of great interest is the development of magnetically separable catalysts (Bronstein and Shifrina, 2011). In this case, MNPs are functionalized and serve as a support for catalytic complexes formation. Such catalytic MNPs have unique catalytic properties through the high surface area and consequently the increase of active sites number (Baird et al., 2016). The immobilization of enzymes on magnetic nanoparticles is an upcoming trend because these nanoparticles have a high surface area and provide easy separation from the reaction mixture (Akhond et al., 2016). In the work (Pieters and Bardeletti 1992), magnetic particles were used for immobilization of GOx and for their functionalization by amino groups, poly(ethylene imine) (PEI) was used. In this paper, 3-aminopropyl) trimethoxysilane is used for MPs surface modification.

In the present work, we developed sustainable D-glucose oxidation with magnetically separable biocatalyst. For the first time, the method of synthesis of a magnetically separated catalyst based on immobilized GOx and MPs is proposed. This biocatalyst was synthesized by coprecipitation, which is coated with a layer of silica modified with amino groups. The GOx covalent attachment has been carried out via the reaction with a glutaric dialdehyde. The exceptional catalytic activity of the biocatalyst developed to make them promising for practical applications.

2. Experimental

2.1 Materials

Iron (III) chloride hexahydrate (99%), iron**(**II**)** sulfate heptahydrate (99%), sodium hydroxide (98%), tetraethyl orthosilicate (TEOS, 99%), glucose oxidase lyophilized powder (*Aspergillus niger,* 174.9 U/mg), glutaraldehyde solution (GA, 25%) and 10X PBS buffer were purchased from Sigma-Aldrich and used as received. APTES (98%) was purchased from Fluka and used without purification. Ethanol was received from Pharmco-Aaper and used as received.

2.2 Synthesis of Iron Oxide Magnetite Particles (MPs)

The synthesis of iron oxide Fe_3O_4 MPs was carried out according to the procedure of classical synthesis by coprecipitation (Laurent et al., 2008). The coprecipitation technique is probably the simplest and most efficient chemical pathway to obtain magnetic particles. Iron oxides (either Fe_3O_4 or γFe_2O_3) are usually prepared by an aging stoichiometric mixture of $FeCl_3 \times 6H_2O$ and $FeSO_4 \times 7H_2O$ in an aqueous medium.

2.3 Functionalization of Fe_3O_4 MPs with TEOS and APTES

Coating of Fe_3O_4 MPs with a silica shell by TEOS treatment was carried out according to the published procedure (Podolean et al., 2013). MPs coated with silica were separated from the reaction solution and washed with water and ethanol, using magnetic separation. Fe_3O_4/Sil particles were suspended in APTES aqueous solution. After that, glycerol was added. The reaction was carried out under stirring at 90 °C for 5 h. Then, the support Fe_3O_4/Sil/Amin was washed and dried in a vacuum oven overnight.

2.4 Attachment of GA and GOx (Biocatalyst - Fe_3O_4/Sil/Amin/Glut/GOx)

Dry Fe_3O_4/Sil/Amin was added to the glutaraldehyde (GA) solution in the PBS buffer (pH 7.0) and stirred for 1 h. The support functionalized with GA was magnetically separated and washed with water. At the same time, GOx was incubated and stirred in the PBS buffer for 1 h. The GOx solution was added to the Fe_3O_4/Sil/Amin/Glut support and stirred for 1 h. Then the biocatalyst was magnetically separated.

2.5 Characterization

Electron-transparent specimens for transmission electron microscopy (TEM) were prepared by placing a drop of a sample suspension onto a carbon-coated Cu grid. Images were acquired at an accelerating voltage of 80 kV on a JEOL JEM1010 transmission electron microscope. The images were analyzed with an image-processing package ImageJ (the National Institute of Health) to estimate nanoparticle diameters.
Magnetic measurements were performed on a Quantum Design PPMS-14 magnetometer using the systems DC measurement capabilities. Milligram quantities of the sample were placed in a standard gelatin capsule.
X-ray powder diffraction (XRD) patterns were collected on an Empyrean from PANalytical. X-rays were generated from a copper target with a scattering wavelength of 1.54°A. The step size of the experiment was 0.02.

2.6 D-glucose Oxidation and Product Analysis

D-glucose oxidation was carried out at atmospheric pressure in the temperature range of 30 - 50 °C in a double-jacketed three-neck round-bottom flask equipped with a gas inlet, overhead stirrer, and a reflux condenser, serving as a gas outlet. The reaction temperature was maintained by circulating a heating medium in the flask jacket. After reaching the required temperature, 15 mL of the 0.1 M phosphate buffer (pH 7.2), 10 mg of D-glucose and 0.11 g of the biocatalyst were loaded in the flask and kept stirring for 60 min. Oxygen with a feeding rate of 440 – 450 mL/min was used as the oxidant. After the reaction, the catalyst was separated with a rare-earth magnet and the reaction mixture was analyzed using HPLC, UltiMate 3000 (ChromaTech, Russia) equipped with a refractometry detector and a ReproGel H Column (500x10 mm, NTP 160000). The H_2SO_4 solution (9 mM) was used as eluent at the 0.5 mL/min rate for 30 min at the eluent pressure of 6.5 kPa and the column temperature of 250°C. Pure D-gluconic acid was used for product identification.

3. Result and Discussion

Magnetic iron oxide particles have been synthesized in a co-precipitation process. The Fe_3O_4 MPs were coated with a silica layer (Fe_3O_4/Sil), functionalized with amino groups via the APTES attachment (Fe_3O_4/Sil/Amin) followed by the binding of the GA linker to create aldehyde groups on the support for the GOx attachment (Fe_3O_4/Sil/Amin/Glut/GOx). This method is known to form iron oxide nanoparticles which tend to aggregate into big particles (Jadhav et al., 2013). Changing an iron precursor concentration, we were able to vary the Fe_3O_4 MPs size. The optimal concentrations of iron precursors ($FeSO_4 \cdot 7H_2O$ and $FeCl_3 \cdot 6H_2O$) are 0.038M and 0.076M respectively. The iron oxide MPs of 160±40 nm in diameter are shown in Figure 1(a). These TEM images of Fe_3O_4/Sil/Amin and Fe_3O_4/Sil/Amin/Glut/GOx (Figure 1(b, c)) show the presence of modifiers and enzyme on the surface of iron particles.
A representative XRD pattern of Fe_3O_4 MPs (Figure 1d) shows a set of reflections which are characteristic of a spinel structure, most probably magnetite, Fe_3O_4 according to the standard card (JCPDS 19-629). The magnetization curves for Fe_3O_4 MPs samples presented in Figure 2 (a) demonstrate that the saturation magnetization value is 23,69 emu/g. This value agrees to bulk magnetite.
The results of the study of the properties of the biocatalyst (Fe_3O_4/Sil/Amin/Glut/GOx) during the oxidation of D-glucose to D-gluconic acid are presented in Table 1. The activity of the biocatalyst was evaluated by the yield of D-gluconic acid, taking into account the fact that the native enzyme exhibits a selectivity of 100% (D-gluconic acid yield of 100%, respectively).
To study the D-glucose concentration of influence on its oxidation, the experiments were performed in the D-glucose concentration range of 0.46 - 5.52 mmol/L (**#** 1-7 Table 1) at T 40 **°C** and pH 5 for better comparison with native GOx. From the presented data it is seen that with increasing concentration of D-glucose, D-gluconic acid production was increased. The optimal initial D-glucose concentration was 3.68 mmol/L and D-gluconic acid yield was 88%. This activity of the biocatalyst is high enough for immobilized GOx and comparable with the literature data. The dependence of the biocatalyst activity on the temperature is presented in Table 1 (#5, 8-11). To study the temperature influence on the D-glucose oxidation, the experiments were performed in the temperature range of 30-50 °C. The native enzyme shows the maximum activity at 40 °C, followed by a sharp decrease in the product yield, indicating a loss of the enzyme activity

most likely due to denaturation at higher temperatures. In the case of immobilized GOx (for Fe_3O_4/Sil/Amin/Glut/GOx), we observed the lower D-gluconic acid yield at 30-40 °C as compared to that of native GOx. This is consistent with prior findings and due to some loss of mobility and reactivity of GOx after immobilization (Pieters et al., 1992). On the other hand, the further temperature increase to 50 °C resulted in much higher product yields (by 15-20%) with the immobilized catalysts than that with the native enzyme (Golikova et al., 2017).

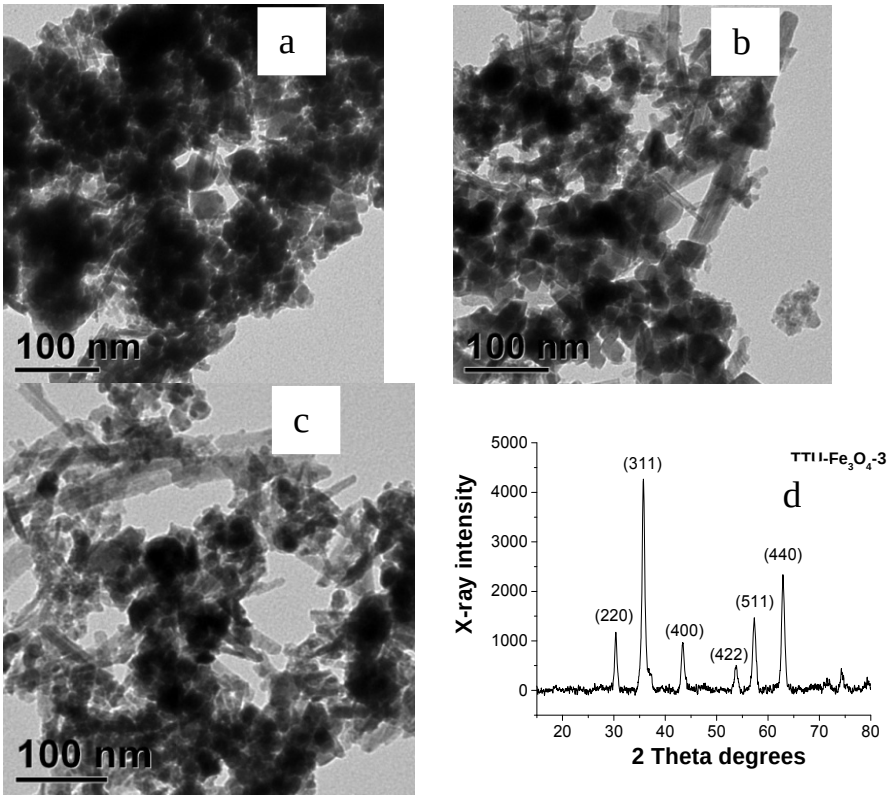

Figure 1: TEM images of Fe_3O_4 MPs (a), Fe_3O_4/Sil/Amin (b) and Fe_3O_4/Sil/Amin/Glut/GOx (c) and XRD pattern of Fe_3O_4 MPs (d).

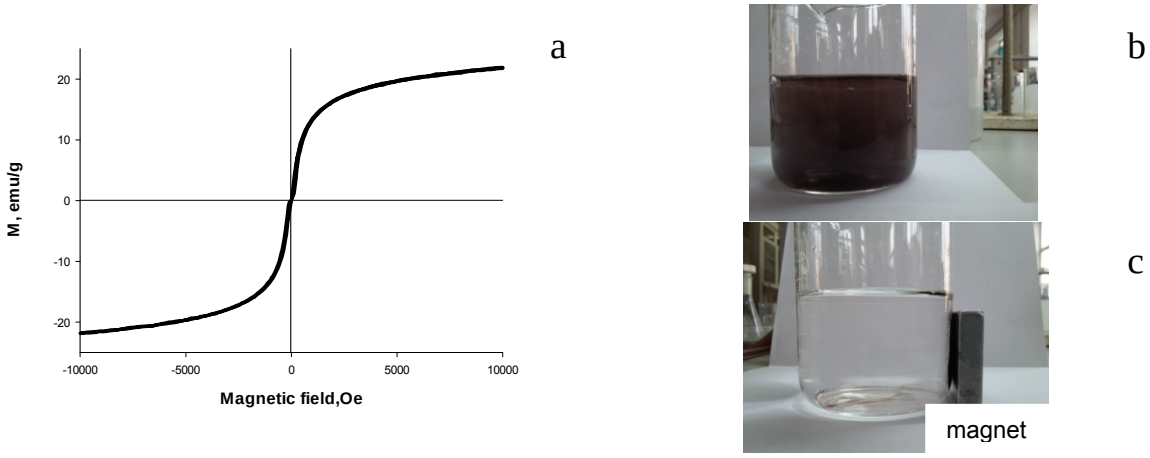

Figure 2: *Magnetization curves of Fe_3O_4 MPs(a), the reaction solution before the magnet separation from Fe_3O_4/Sil/Amin/Glut/GOx (b), the reaction solution after the magnet separation from Fe_3O_4/Sil/Amin/Glut/GOx (c)*

To study the reaction time of influence on the D-gluconic acid yield, the experiments were performed in the reaction time range of 30 – 120 min (# 5, 12-14, Table 1). With increasing reaction time up to 60 minutes, D-gluconic acid yield was increased to 88%. With a further increase in the reaction time to 120 minutes yield of D-gluconic acid is practically unchanged.

Table 1: D-gluconic acid yield depending on the conditions of D-glucose oxidation in the presence of a biocatalyst Fe_3O_4/Sil/Amin/Glut/GOx

No	Concentration of D-glucose, mmol/L	Reaction time, min	Temperature, °C	D-gluconic acid yield, %
1	0.46	60	40	19.4
2	0.92	60	40	42.2
3	1.84	60	40	50.6
4	2.76	60	40	63.3
5	3.68	60	40	88.0*
6	4.60	60	40	78.5
7	5.52	60	40	71.7
8	3.68	60	30	72.8
9	3.68	60	35	78.1
10	3.68	60	45	86.8
11	3.68	60	50	84.4
12	3.68	30	40	63.5
13	3.68	90	40	87.5
14	3.68	120	40	87.3

* *D-gluconic acid yield during D-glucose oxidation in the presence of the native enzyme is 100%*

As noted above, the biocatalyst (Fe_3O_4/Sil/Amin/Glut/GOx) obtained on the basis of GOx immobilized on MPs have magnetic properties and can be easily separated by a magnet from the reaction product (Figure 2 (b, c)). This is a great advantage for practical applications compared with the same enzymes immobilized on inorganic supports (SiO_2 and Al_2O_3). A comparison of the biocatalysts based on SiO_2 and Al_2O_3 with magnetically separable biocatalyst showed that in the case of the Fe_3O_4/Sil/Amin/Glut/GOx in gluconic acid yield was higher.

4. Conclusions

We developed magnetically separable biocatalyst (Fe_3O_4/Sil/Amin/Glut/GOx) by tethering GOx via a GA linker to the MPs coated with silica and modified by APTES. The MPs were synthesized by coprecipitation and consist of magnetite particles of 160±40 nm in diameter, which demonstrate a strong magnetic response and very fast magnetic separation. The higher thermolability (50 ºC) of the designed biocatalyst as compared to native GOx (optimal temperature 30-40 ºC) was shown. A comparison of the biocatalysts based on SiO_2 and Al_2O_3 with magnetically separable biocatalyst showed that in the case of the Fe_3O_4/Sil/Amin/Glut/GOx in gluconic acid yield was higher (88%). The high catalytic activity of the developed magnetically separable biocatalyst (Fe_3O_4/Sil/Amin/Glut/GOx) performance make this biocatalyst promising for practical applications.

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