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Bienzyme Biocatalysts Based on Mesoporous Silica in a Cascade Reaction

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A cascade reaction is defined as the chemical process which consists of two or more consecutive reactions carried out in the same reaction vessel due to the multifunctionality of the catalyst. This approach was utilized by researchers to design advanced biocatalysts with two or more enzymes immobilized on the same support. The key benefits of multienzyme biocatalysts rise from a single pot, where consecutive reactions are performed, thus eradicating the handling of intermediate products. This makes processes more sustainable, allowing one to obtain target molecules in a more environmentally friendly way. We report on the effect of pore size in biocatalysts based on mesoporous silica on the loading of immobilized enzymes glucose oxidase (GOx) and horseradish peroxidase (HRP) as well as on the activity and the possibility of reuse of the bio-enzyme biocatalyst in a cascade reaction. The choice of silica substrates was due to their commercial availability and sharply different pore sizes: 6 nm and 15 nm. In the first case, the pores are too small to accommodate GOx and HRP, the hydrodynamic diameter of which is ~ 8 nm for each, while in the second case, there is enough space for immobilization of GOx and HRP inside the pores. We demonstrate that larger pores provide higher enzyme throughput due to immobilization within the pores, remarkable 95% relative activity, and increased stability during reuse due to the conformational integrity of GOx and HRP in spacious pores. This study clearly shows the advantages of substrates with pores larger than the size of the enzyme for its immobilization during the synthesis of the biocatalyst.

1. Introduction

In recent decades, cascade biocatalysis has aroused considerable interest from both the scientific community and industry as a promising technology for environmentally friendly and sustainable chemical production. Unlike step-by-step synthesis, cascade biocatalysts carry out two or more consecutive reactions in “one-pot”, which avoids the isolation and purification of intermediates (Braz et al., 2023). This procedure has become more effective by reducing the reaction time and production costs while no toxic waste is generated (Ren et al., 2019). Currently, numerous drugs and fine chemicals, including chiral alcohols and amines, carbohydrates, and polymers, are synthesized by enzymatic cascade catalysis. However, the industrial use of cascade biocatalysts based on free enzymes is still problematic due to their low stability and complexity of reuse (Arana-Pena et al., 2021).

The use of a polymer, poly (acrylic acid), for covalent conjugation of glucose oxidase (GOx) and horseradish peroxidase (HRP) with subsequent adsorption on graphene oxide was presented by Zore et al. (2015). In work (Pitzalis et al., 2017), covalent immobilization of GOx and HRP on the amino-functional mesoporous SBA-15 was proposed, providing increased thermal stability and recyclability during detoxification of agricultural wastewater containing both glucose and recalcitrant phenolic compounds. Two- or three-stage cascade reactions were carried out with multi-enzyme films formed by self-assembly of protein-polymer surfactants containing GOx and HRP, or β-glucosidase (Farrugia et al., 2017). Despite some loss of catalytic activity compared to native enzymes, the ease of manipulating biocatalyst films, as well as the possibility of using the function of chemical reactions by easily changing the location of the film makes these multi-enzyme

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biocatalysts promising as controlled multifunctional biomaterials. Reverse opal hydrogel particles were used as a carrier for the immobilization of HRP and urease (Wang et al., 2017). The assembly of particles created an ideally ordered porous structure for the immobilization of enzymes, providing easy penetration of substrates. Due to the ordering, this carrier has unique photonic forbidden zones, which lead to bright colors for encoding immobilized enzymes. Encoded carriers with various immobilized enzymes could be mixed in various ways to conduct cascade reactions. Despite these structural achievements, the activity of this catalyst did not exceed 50% of the activity of native enzymes, which indicates that there are opportunities for improvement. Crosslinking of GOx and HRP with the DNA scaffold by self-assembly and encapsulation of this structure in MOF based on zeolite imidazolate scaffold-8 (ZIF-8) allowed reducing the losses of the biocatalyst enzyme by ten times compared with conventional encapsulation in MOF (Song et al., 2019). This biocatalyst first oxidized glucose to form H_2O_2, followed by the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)$^{2-}$ ($ABTS^{2-}$) HRP with the formation of $ABTS^{-}$. It has demonstrated excellent catalytic efficiency, stability and reusability due to encapsulation in the DNA framework. There are several examples where magnetic carriers have been used for cascade reactions. A magnetic bienzyme biocatalyst was synthesized by covalent binding of α-amylase and glucoamylase to the NPS Fe_3O_4/SiO_2 core-shell for single-use starch hydrolysis (Bian et al., 2018). It is noteworthy that a significant increase in the activity of the biocatalyst was achieved by using a protective film formed by the coordination of Fe^{3+} ions with multifunctional tannic acid to protect enzymes from denaturation and detachment. Using a similar magnetic substrate, but using DNA modification before the addition of GOx and HRP to obtain enzyme-DNA conjugates (Song et al., 2018), the authors were able to significantly improve the catalytic properties compared to mixtures of free enzymes and biocatalysts formed as a result of nonspecific adsorption on the substrate.

An interesting example of the use of a bienzyme biocatalyst was presented by Rezaei et al. (2019). GOx and glucoamylase were covalently attached to aminofunctional Ag-dendrites (prepared in the presence of Ni Raney catalyst), creating a large surface area for the immobilization of the enzyme. The catalyst was tested during the cascade conversion of starch into gluconic acid and showed better activity and stability over a wide range of pH and temperatures compared to native enzymes. Surprisingly, however, despite the fact that Ag can also play a catalytic role in combination with enzymes, there is no discussion of the effect of Ag dendrites on catalysis in the reference (Rezaei et al., 2019), as well as a comparison with non-metallic carriers.

Mesoporous silica is a promising material for a number of technological applications due to its outstanding properties (Pitzalis et al., 2017). In particular, the use of mesoporous silica as an enzymatic carrier for immobilized biocatalysts seems to be a very appropriate application (Braz et al., 2023). Covalently bound enzymes are more resistant to harsh reaction conditions and can be reused over several reaction cycles (Sulman et al., 2019).

Glucose oxidase (GOx; EC.1.1.3.4) and Horseradish peroxidase (HRP; EC. 1.11.1.7) - these are two universal redox enzymes that are used in biosensorics, tissue engineering and are very promising candidates for green chemistry and bioremediation. GOx oxidizes glucose to gluconolactoneand reduces oxygen to H_2O_2, using flavinadenine dinucleotide as a cofactor. HRP is a heme protein that uses H_2O_2 to oxidize various aromatic substrates. HRP is an enzyme that is activated by high concentrations its own substrate (H_2O_2) (Pitzalis et al., 2017). Hence, HRP activity could would save by producing in situ a controlled amount of H_2O_2. This involves the joint use of GOx and HRP to create a bio-enzymatic biocatalyst.

In our preceding work, we used the 6 nm pores of commercial mesoporous silica followed by a covalent attachment of glucose oxidase (GOx) (Jaquish et al., 2018). The choice of the commercial silica further simplified the procedure and reduced the cost of the biocatalyst fabrication. However, 6 nm pores in this catalyst are smaller than the GOx size (hydrodynamic diameter is 7.6 nm) and HRP (hydrodynamic diameter is 8.0 nm), thus, eliminating the possibility of the enzyme attachment and catalytic reaction inside the pores. In the present work, we studied the performance of the bienzyme biocatalysts based on silica with 6 nm and 15 nm pores. We demonstrate that the bienzyme biocatalyst based on the silica with 15 nm porous displays remarkable in cascade reactions.

2. Experimental

2.1 Materials

Silica gel (99%, 6 nm pores, 200-425 mesh, designated 6nm-SiO_2), silica gel (99%, 15 nm pores, 200-425 mesh, designated 15nm-SiO_2), D-glucose (99.5%), Peroxidase from horseradish, lyophilized powder (HPR, 173 U/mg), glucose oxidase (GOx) lyophilized powder (*Aspergillus niger,* 174.9 U/mg), phosphate buffered saline (PBS buffer), and glutaraldehyde solution (GA, 25%) were purchased from Sigma and used without purification. The amino group containing silane (3-aminopropyl)triethoxysilane, APTES, 98%) was acquired from Fluka and used as received. ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium

salt), 98%) was obtained from TCI and used as received. Ethanol was purchased from Pharmco-Aaper and used as received.

2.2 *Synthesis of biocatalysts by GOx immobilization*

Silica supports, 6nm-SiO_2 and 15nm-SiO_2, were functionalized with amino groups using APTES (Jaquish et al., 2018). The last step of the biocatalyst preparation included a treatment with GA (a linker) in the PBS buffer followed by the interaction with the GOx-HRP solution. Two separated solutions of GOx and HRP both, at the concentration 2 mg/mL, were prepared by dissolving the enzymatic powders in 0.1 M phosphate buffer at pH 8.0. The GOx solution (2 mg/mL) and the HRP solution (2 mg/mL) were mixed together to obtain a mass ratio GOx:HRP = 1:1. Then, the resulting bi-enzymatic solution was added to the glutaraldehyde-activated silica support, and leftunder stirring at 25 °C for 48 h. The biocatalysts obtained were designated 6nm-SiO_2-GOx@HRP and 15nm-SiO_2-GOx@HRP.

2.3 *Biocatalyst activity assays*

The catalytic activity of the bi-enzyme biocatalysts synthesized was measured using the cascade reaction of D-glucose oxidation to D-gluconic acid in the presence of ABTS, allowing spectrophotometric measurements (Figure 1) (Memon et al., 2019).
In the presence of oxygen, GOx catalyzes D-glucose oxidation to first equimolar amounts of D-glucono-1,5-lactone and H_2O_2. Then D-glucono-1,5-lactone is spontaneously hydrolyzed to D-gluconic acid (Figure 1). To assess the activity of GOx, we quantitatively determined the amount of hydrogen peroxide (which matches the amount of D-glucose in moles) at certain time intervals using spectrophotometry. For this, we added a chromogenic compound, ABTS (Figure 1), which is oxidized to a colored compound (Figure 2) when H_2O_2 is decomposed by HRP.

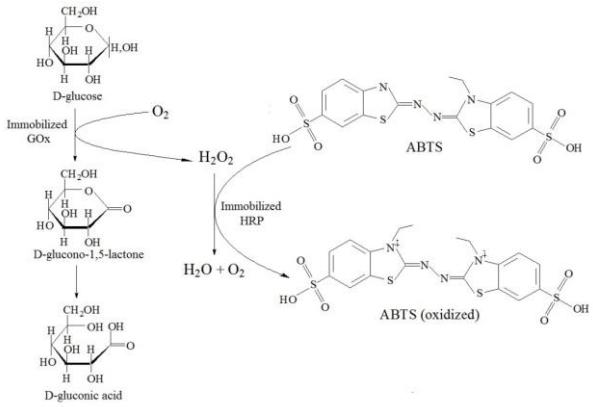

Figure 1: Schematic representation of cascade reaction: D-glucose oxidation to D-glucono-1,5-lactone and decomposed of H_2O_2 in the presence of bi-enzyme biocatalyst and ABTS

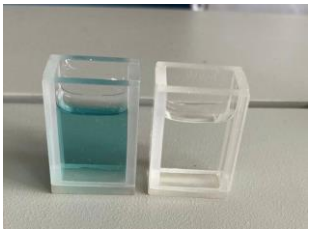

Figure 2: Left cuvette shows the reaction solution after the ABTS oxidation, while the right cuvette shows the solution before that

In a typical experiment, 1.5 mL of D-glucose (50 mM), 1.5 mL of ABTS (0.02 M), and 15 µL of native GOx and HRP (0.5 mg) solution or 0.05 g of the bi-enzyme biocatalyst containing 0.5 mg of GOx and HRP were mixed with 1.8 mL of the PBS buffer at pH 6.0. Then the solutions were incubated for five minutes, after which the absorbance was measured using a UV-5 spectrophotometer (UV/VIS Mettler Toledo) at 415 nm. It is noteworthy that in the enzyme activity measurements both native and immobilized GOx samples had the

same concentration of GOx and each experiment was repeated three times. The activity was determined by the amount of oxidized glucose, which is equimolar H_2O_2. The relative activity of immobilized enzymes was calculated as a percentage of the retained activity of native enzymes.

2.4 *Characterization*

Nitrogen adsorption measurements were carried out at liquid nitrogen temperature on an ASAP 2020 analyzer from Micromeritics. Samples were degassed at 100 °C in vacuum. The total surface area was estimated by the Brunauer–Emmett–Teller (BET) method, while the pore size distribution was determined by the Barrett–Joyner–Halenda (BJH) method using desorption.

3. Results and discussion

Figure 3 (a, c) and Table 1 show the liquid nitrogen adsorption data for parent silica samples and two bi-enzyme biocatalysts. For both samples, the adsorption-desorption isotherms represent type IV, commonly observed for mesoporous materials. The BET (Brunauer-Emmet-Teller) method was used to estimate the total surface area. For 6nm-SiO_2-GOx@HRP, it measures 356 m^2/g, while for 15nm-SiO_2-GOx@HRP, it is 197 m^2/g which is consistent with the larger pores of this sample. A comparison of porosity of the enzymes silica with those of the parent silica samples (Figure 3 b, d and Table 1) demonstrates that the total surface area decreases in both cases after immobilization of enzymes. A decrease in the surface area is observed because of the enzymes molecules with a mean size of 6-8 nm are deposited in the pores of the support (for pores with the size of 15 nm) and on the surface (in the case of pores with the size of 6 nm) partially blocking them. The pore size distributions, however, stay practically unchanged in both cases, revealing that the majority of smaller pores are not blocked.

Table 1: BET surface areas and pore volumes of bi-enzyme biocatalysts and parent silica samples

Sample notation	BET surface area, m^2/g	BET pore volume, cm^3/g
6nm-SiO_2	458	0.73
6nm-SiO_2-GOx@HRP	356	0.51
15nm-SiO_2	255	1.02
15nm-SiO_2-GOx@HRP	197	0.74

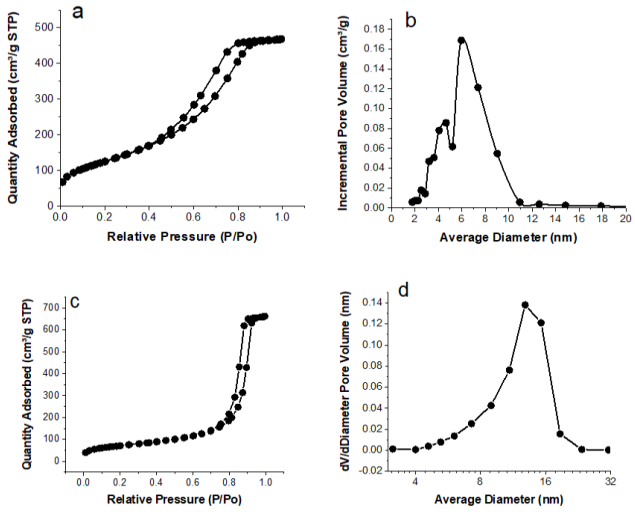

Figure 3: N_2 adsorption-desorption isotherms (a, c) and pore sizes distributions (b, d) of 6nm-SiO_2 (a, b) and 15nm-SiO_2 (c, d)

Several steps were performed for covalent attachment of GOx and HRP to the initial silica carriers. First, silica was treated with APTES for their functionalization by amino groups. Then the amino groups of the carriers reacted with GA, after which the aldehyde groups of GA reacted with the amino groups of GOx and HRP. To quantify how much GOx was immobilized in each case, the biocatalyst was separated from the filler fluid after GOx immobilization and the catalytic activity of the filler fluid was evaluated. This allowed us to determine the immobilization coefficient (IC) for each biocatalyst. Thus, in the case of a 6 nm SiO_2 support, the

immobilization coefficient was 62 %, and for a 15 nm SiO_2 carrier, it was 78 %. This difference is probably due to the fact that large pores are able to accommodate GOx and HRP molecules.
Important result obtained in this work is the long-term stability during incubation of 6 nm-SiO_2-GOx@HRP and 15 nm-SiO_2-GOx@HRP compared to native GOx@HRP. To evaluate this parameter, the activity of all three biocatalysts was periodically measured for 60 h at 25 °C and 60 d at 4 °C. The data presented in Fig. 4a, show that free GOx@HRP retained only 35 % of its activity after 30 h, while the activity of 6 nm-SiO_2-GOx@HP and 15 nm-SiO_2-GOx@HP decreased from 83 % to 53 % and from 95 % to 74 %, respectively. In addition, the activity of native GOx@HRP decreased to 3 % after 60 h, while 6 nm-SiO_2- GOx@HRP and 15 nm-SiO_2-GOx@HRP retained 32 % and 49 % of their activity, respectively. This phenomenon of a decrease in enzyme activity during storage at room temperature was observed earlier and was explained by the decomposition of protein as a result of bacterial growth.

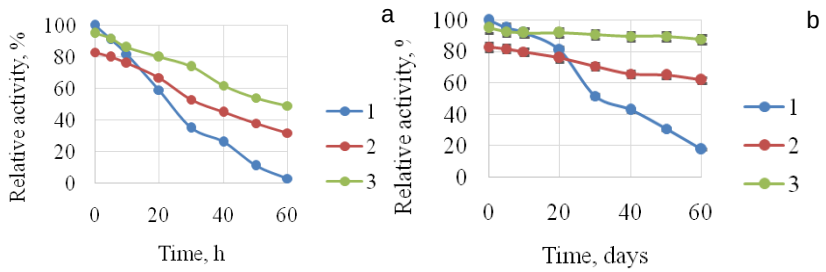

Figure 4: Long-term incubation stability at 25 °C (a) and 4 °C (b) of native GOx@HRP (1), 6nm-SiO_2-GOx@HRP (2) and 15nm-SiO_2-GOx@HRP (3)

In the case of long-term stability at 4 °C, when bacterial growth is prevented (Fig. 4b), the activity of native GOx@HRP remains high for 5 d and decreases to 18 % after 60 d, which can probably be attributed to denaturation. For immobilized GOx@HRP, the activity is more stable over time, but it differs significantly for biocatalysts with different pore sizes. The biocatalyst 6nm-SiO_2-GOx@HRP retained 62 % of its activity after 60 d, while 15nm-SiO_2-GOx@HP demonstrated remarkable stability with 88% activity retention after 60 d. We believe that the best storage stability of 15nm-SiO_2-GOx@HRP is determined by the optimal conformational configuration of the enzyme inside the pores, preventing denaturation and increasing the storage time of GOx@HRP.A higher activity of 15nm-SiO_2-GOx@HRP biocatalyst in comparison with 6nm-SiO_2-GOx@HRP is related to the higher size of the pores of support leading to the higher availability of the active sites of bienzymes (GOx@HRP) to substrates. An increase in the biocatalyst stability is due to the influence of the carrier and the better enzyme immobilization in the pores of SiO_2.
The stability and reusability of immobilized enzymes are two important issues that determine the potential of biocatalysts for commercial use. This is especially important because native enzymes are expensive compared to conventional catalysts. However, if the characteristics of the immobilized enzymes are stable and they can be reused after separation from the reaction mixture (which is especially facilitated in the case of catalysts with magnetic reduction), these factors reduce the cost of enzymes and the cost of the catalyst per unit of production (Memon et al., 2019).
To evaluate the stability of 6nm-SiO_2-GOx@HRP and 15nm-SiO_2-GOx@HRP during repeated use, seven consecutive experiments were conducted using D-glucose oxidation under optimal conditions (pH 6.0 and 40 °C). After each reaction, the biocatalyst was separated from the reaction mixture and used again in the next reaction. Data on the possibility of reuse are shown in Figure 5. For 6nm-SiO_2-GOx@HRP, the relative activity decreases by 8% after seven consecutive cycles, while 15nm-SiO_2-GOx@HRP has lost only 3% of its activity.

4. Conclusions

Two bi-enzyme biocatalysts based on mesoporous silica with 6 nm and 15 nm pores were synthesized to study the influence of pore size on the enzyme loading and biocatalytic activity of 6nm-SiO_2-GOx@HRP and 15nm-SiO_2-GOx@HRP. The choice of silica supports with the above pores was determined by size of GOx and HRP, whose hydrodynamic diameter is ~ 8 nm. Thus, in the case of the 6nm-SiO_2 support, enzyme molecules can be located only outside the pores, while in the case of 15nm-SiO_2, the GOx and HRP molecules can be immobilized inside the pores presumably without loss of conformation integrity. This results in several crucial outcomes for 15nm-SiO_2, such as the higher loading capacity (immobilization coefficient 78 %), remarkable relative activity (95 %) as well as its excellent stability in repeated use (for 15nm-SiO_2-

GOx@HRP, the relative activity decreases by 3 % after seven consecutive cycles). These factors, combined with commercially available source silica carriers, make this biocatalyst promising for successful commercialization.

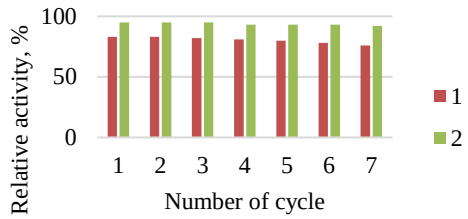

Figure 5: Relative activity of 6nm-SiO_2-GOx@HRP (1) and 15nm-SiO_2-GOx@HRP (2) in seven consecutive cycles

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