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Metal-Enzyme Biocatalyst for the Conversion of Cellulose to Sorbitol

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A new bifunctional nanobiocatalyst for the single cascade conversion of carboxymethyl cellulose (CMC) to sorbitol has been developed. The nanobiocatolyst is based on aggregates of magnetic nanoparticles (MNA) functionalized with chitosan (CS) and crosslinked with tripolyphosphate (TPP). It contains two types of catalytic centres: cellulase (Cel) and ultra-small Pd nanoparticles (NPs). The complete characterization of MNA-CSP-Pd-Cel and preliminary studies allowed us to select optimal conditions and cascade CMC into sorbitol in a yield of 80.4 % for 15 h at a temperature of 70 °C and a hydrogen pressure of 5 MPa. The yield demonstrated in this work is much higher than has been reported so far for the same cascade process.

1. Introduction

Biomass is considered as a carbon-neutral energy source that can be used as a raw material for the production of highly dispersed chemicals. Cellulose is a promising raw material for the production of bioenergy with great potential in the field of environmentally friendly fuels and various biochemical synthesis (Bayu et al., 2019). Biofuels and value-added chemicals such as sorbitol, ethanol, green hydrogen, and 5-hydroxymethylfurfural (HMF) can be produced from cellulose by chemical catalytic processes (Xu et al., 2022). Sorbitol is one of the most valuable chemicals obtained from biomass, which can serve as an intermediate for the synthesis of various drugs, food additives, cosmetics and environmentally friendly hydrogen energy (Zhang et al., 2013).
An environmentally friendly way of processing biomass is the use of enzymes that decompose cellulose to glucose (Arumugam et al., 2021). However, the low thermal stability of enzymes and their storage stability, as well as the presence of impurities, enzyme leakage, and the problem of reuse are the main disadvantages of using free enzymes. These disadvantages can be minimized by immobilizing enzymes on various substrates (Zanuso et al., 2021). The role of carrier materials is to preserve the secondary structure of the enzyme, as well as to create favourable interactions with the enzyme (Zdarta et al., 2018). Cellulases are a cocktail of three enzymes: endoglucanases, exoglucanases, and β-glucosidases, which are used to break various chemical bonds in cellulose (Sulman et al., 2022). For simplicity, here we will use the term “cellulase” (Cel), which means the aforementioned cocktail, unless otherwise indicated. In the last decade, enzymes have been immobilized on nanostructured substrates such as nanoparticles (NPS) of various sizes and shapes, including nanorods and nanofibers (Medina-Castillo et al., 2022), materials with pores in the nanometer range (Zhang et al., 2021), irritant-sensitive nanoparticles, etc.
Magnetic nanoparticles (MNPs), as supporting matrices for the immobilization of enzymes, including cellulase, can certainly be extracted using an external magnetic field. MNPs also have a number of desirable characteristics, for example, a large surface area, which facilitates effective immobilization, high stability, environmental friendliness, reusable, etc (Guillermo et al., 2022). Functionalization of MNPs leads to increased compatibility with the enzyme and, consequently, stability (Jiang et al., 2024). The addition of an inert polymer such as chitosan ensures biocompatibility, biodegradability, non-toxicity and, at the same time, can act as a hydrophilic base (Ahmed et al., 2023).
The integration of heterogeneous chemical and biological catalysis is considered as an important strategy for obtaining chemicals from biomass, especially in cascade-type redox reactions (Sun et al., 2018). The

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combination of enzymes and catalytic centres based on transition metals for the development of multifunctional catalysts has been well demonstrated (Yang et al., 2023). Such catalysts containing both noble and highly earthy metals, as well as enzymes, have been successful in both parallel and sequential cascade reactions (Gonzalez-Granda et al., 2023). The latter method is recommended when the catalysts are incompatible and cannot be combined on a single substrate, but a single multifunctional catalyst is preferable from the point of view of process optimization. Since multifunctional catalysts can simplify multi-stage processes, increase their efficiency and yield of the target product, and simplify the purification and isolation of reaction products, they can reduce the accumulation of intermediate reaction products, thereby reducing the toxicity caused by excessive local concentration of intermediate products.

In this work, the combination of Pd nanoparticles with Cel on the same functionalized magnetic substrate leads to the creation of a new bifunctional nanobiocatalyst, the properties of which were studied during the chemoenzymatic cascade conversion of carboxymethylcellulose (CMC) to D-sorbitol in a single reactor. Using transmission electron microscopy (TEM), high-resolution TEM (HRTEM) images, as well as energy-dispersive scanning TEM (EDS), X-ray photoelectron spectroscopy (XPS), infrared spectra of samples, and X-ray powder diffraction (XRD), the relationship between the structure and bonding of catalysts was established.

Materials and methods

Iron (III) chloride hexahydrate (99 %) ($FeCl_3 \cdot 6H_2O$), iron (II) chloride tetrahydrate (99 %) ($FeCl_2 \cdot 4H_2O$), ammonium hydroxide solution (25 %), citrate buffer solution (CBS buffer), phosphate buffered saline (PBS buffer), and sodium tripolyphosphate (TPP) were purchased from Nevareaktiv (Russia) and used without purification. Palladium (II) acetate ($Pd(CH_3COO)_2$) was purchased from Sigma-Aldrich and used without purification. Cellulase (Cel) lyophilized powder (EC 3.2.1.8, Aspergillus niger, 50 U/mg) was purchased from Biopreparat (Russia) and used as received. Chitosan middle-viscous, insoluble matter < 1 %, 400 KDa and N-(3-dimethylamiopropyl)-N'-ethyl carbodiimide hydrochloride (EDC, 99 %) were obtained from FLUKA, BioChemika (Japan). N-hydroxysuccinimide (NHS, 98 %) was purchased from Acros Organics (China). 3,5-Dinitrosalicylic acid (DNS) was purchased from Pallav (India). Carboxymethylcellulose (CMC) sodium salt was purchased from Juning Fortune Biotech (China).

The synthesis of MNA was carried out according to the procedure published elsewhere using $FeCl_2$ and $FeCl_3$ and co-precipitating them in basic conditions (Matveeva et al., 2024). Coating of MNA with CS followed by further cross-linking with TPP is reported in our preceding paper (Matveeva et al., 2024). The sample synthesized was labelled MNA-CSP.

For the synthesis of MNA-CSP-Pd, palladium acetate solution was prepared by stirring for 15 min 0.073 g $Pd(CH_3COO)_2$ in 20 mL of tetrahydrofuran, after which the mixture was heated to 60 °C, MNA-CSP particles were added and the mixture was left to mix for 24 h at 60 °C to ensure coordination of Pd ions with chitosan amino groups. After that, the particles were washed several times with distilled water and extracted using a permanent magnet. Next, a $NaBH_4$ solution was prepared (0.06 g in 50 mL of distilled water) while cooling by ice. 25 mL of $NaBH_4$ solution was mixed with the particles while cooling on ice, stirred for another 10 min, after which, with constant stirring, the remaining 25 mL of $NaBH_4$ solution was added drop by drop and stirred for another 10 min. After that, the particles were washed several times with distilled water, extracted using a permanent magnet, and dried in air.

According to the elemental analysis data, the palladium content in the MNA-CSP-Pd is 3.1 %.

For the covalent attachment of Cel on the support, we used EDC and NHS. When these reagents are added and kept in the reaction mixture for 12 h, a stable NHS ester is formed on the surface of Cel. In a typical experiment, the dry MNA-CSP-Pd sample (1.0 g) was added to 0.1 g of EDC, 0.04 g of NHS, and 50 mg of Cel dissolved in 25 mL of citrate buffer (pH 5.0) and stirred for 12 h. Then the biocatalyst (MNA-CSP-Pd-Cel) was magnetically separated, washed five times with 50 mL of water each, and dried at 20 °C for 24 h.

Catalytic efficiency of immobilized Cel was determined according to the original reducing sugar analysis method (DNS assay) (Vasilescu et al., 2022). In a typical experiment, 5 mg of native or immobilized cellulase (0.1 g MNA-CSP-Cel) was incubated with 0.25 % (w/v) of CMC in 25 mL of the 0.1 M sodium citrate buffer with pH 5.0. The reaction mixture was kept under constant stirring for 30 min at 50 °C. The D-glucose released was measured using the UV-5 spectrophotometer (UV/VIS Mettler Toledo) at 540 nm by the DNS method and expressed as glucose equivalent using a standard calibration curve.

To study the catalytic properties MNA-CSP-Pd-Cel the following procedure was used. All one-pot cascade processes were performed in a 100 mL stainless steel autoclave (Parr reactor (Series 5,000 Multiple Reactor) under vigorous stirring (1,000 rpm). In a typical experiment, MNA-CSP-Pd-Cel (0.1 g) was incubated with 0.25 % (w/v) CMC in 25 mL of a sodium citrate buffer with pH 7.0 and the reactor was purged with hydrogen four times to remove air. The reactor was heated to 70 °C and kept at the hydrogen pressure of 5 MPa for a 5 h, 10 h, or 15 h. Samples of the reaction mixture were taken for analysis regularly and analyzed by HPLC.

The cellulose conversion, and the D-sorbitol yield were calculated by the Eq(1) and Eq(2):

$$CMC\ Conversion\ (\%) = \frac{(\text{initial CMC weight} - \text{CMC weight after the reaction})}{\text{initial CMC weight}} \cdot 100\ \% \quad (1)$$

$$D - sorbitol\ yield\ (\%) = \frac{\text{moles of D} - \text{sorbitol}}{\text{moles of D} - \text{glucose units in CMC}} \cdot 100\ \% \quad (2)$$

For reusability experiments, MNA-CSP-Pd-Cel was separated with a rare earth magnet after each catalytic reaction and triple washed using a sodium citrate buffer at pH 7 in order to remove the remaining substrate that could be attached to the support surface. Finally, the biocatalyst was added to the next reaction mixture.
Transmission electron microscopy (TEM) and high resolution (HRTEM) images as well as energy scanning TEM dispersive spectroscopy (EDS) were acquired on Osiris at 200 kV. The samples were prepared by placing a drop of the material suspension on the carbon coated TEM grid.
Magnetization measurements of the samples were carried out in a home-built vibrating sample magnetometer (VSM) with accuracy better than ±0.01 emu/g, which allows measuring bulk and powder samples of 0.01-150 mg in the temperature range of 80-1,000 K and magnetic field of 0-2.5 T.
X-ray photoelectron spectroscopy (XPS) data were obtained using MgKα (hu = 1253.6 eV) radiation with the ES-2403 spectrometer (Institute for Analytical Instrumentation of the RAS, St. Petersburg, Russia) equipped with an energy analyzer PHOIBOS 100-MCD5 (SPECS, Berlin, Germany) and X-ray source XR-50 (SPECS, Berlin, Germany). The infrared spectra of the samples were analyzed using an infrared spectrophotometer with Fourier transform and IRPrestige-21 diffusion reflection prefix (Shimadzu, Japan). X-ray powder diffraction (XRD) was carryout out on Rigaku MiniFlex600 (Rigaku Corporation, Japan) using CuKα-radiation (40 kV, 15 mA, and Ni-Kβ filter) in the angle range 2θ = 10 – 90 º with the step of 0.02 º and the rate of 0.5 °/min.

2. Results and Discussion

2.1 Characterization of MNA-CSP-Pd-Cel and MNA-CSP-Pd

The TEM image shows that large aggregates of MNAs consist of smaller nanoparticles attached to each other (Figure 1a). The formation of aggregates of magnetite nanoparticles is characteristic of the deposition method, which is carried out in the absence of effective stabilizing molecules. We estimated the average particle size using the grain crossing method. To do this, we used about 30 particles in each sample. The aggregates are relatively large (~up to several microns) and polydisperse. The sizes of individual magnetite nanoparticles range from 5 to 30 nm with an average diameter of about 12 nm.

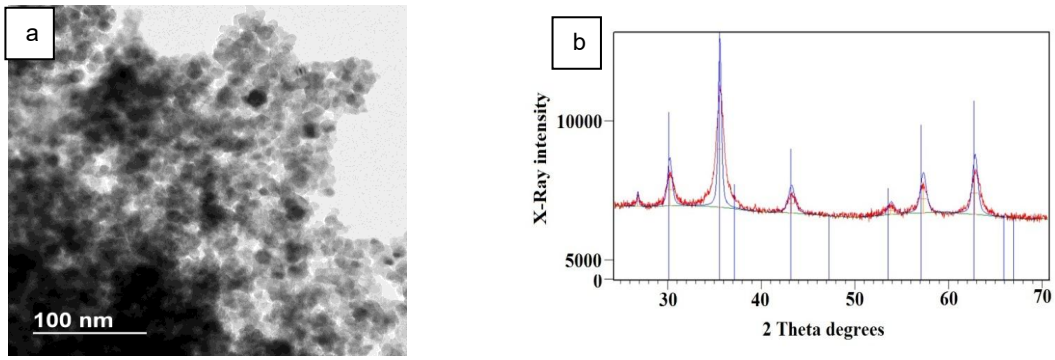

Figure 1: TEM image (a) and XRD profile (b) of MNAs

The X-ray profile of MNAs is shown in Figure 1b. It contains a set of clearer Bragg reflections, the intensity and location of which are typical for magnetite (Fe_3O_4). The average size of MNAs is 25-60 nm, which is consistent with TEM data.
High-resolution TEM (HRTEM) images of MNA-CSP-Pd-Cel are presented in Figure 2a. The HRTEM image clearly shows very small darker spots on the MNA background which could be assigned to Pd NPs. An enlarged image showing the crystal lattice of palladium particles is presented. The average particle size of Pd NPs is 1.0 nm. Hysteresis loops (Figure 2b) demonstrate that the saturation magnetization of MNA-CSP-Pd-Cel (34.0 emu/g) is only 15 % lower than that of parent MNA (40.0 emu/g), revealing that the catalysts can be easily magnetically separated during synthesis and after the catalytic reaction.

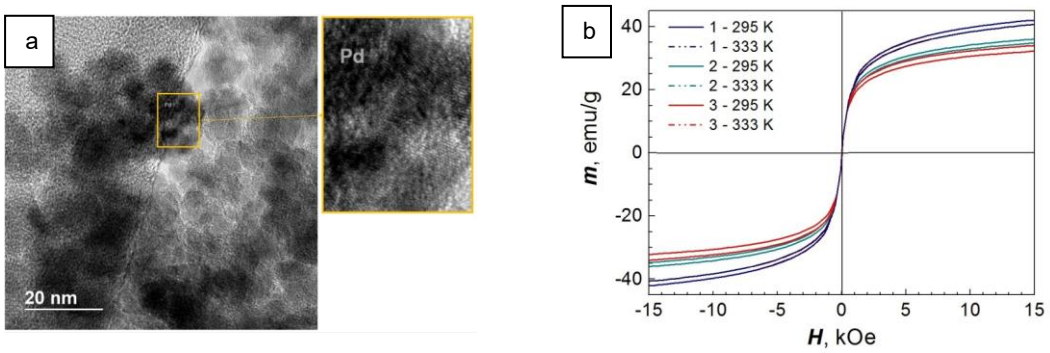

Figure 2: HRTEM images (a) of MNA-CSP-Pd-Cel (yellow square point at Pd NPs) and hysteresis loops (b) of MNA (1), MNA-CSP (2), and MNA-CSP-Pd (3)

Scanning TEM (STEM) energy dispersive spectroscopy (EDS) maps are presented in Figure 3. The maps of individual elements as well as superposition of Fe, P, and Pd maps show that all elements are evenly spread on MNA.

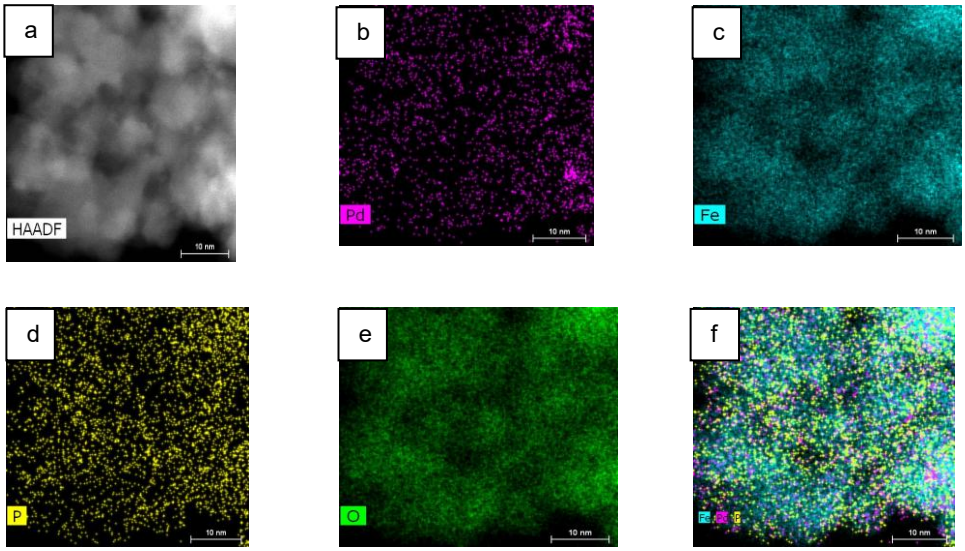

Figure 3: HAADF (high-angle annular dark-field) image (a), and STEM EDS maps of Pd (b), Fe (c), O (d), and P (e) in MNA-CSP-Pd-Cel. Superposition of the Fe, P, and Pd maps is shown in (f)

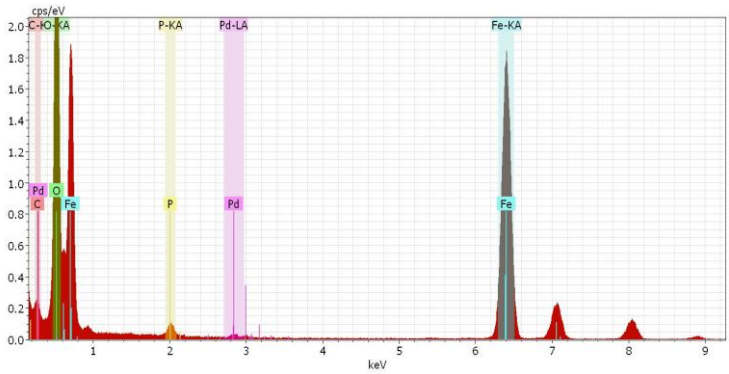

Figure 4: EDS spectrum of MNA-CS-Pd-Cel

The EDS spectrum presented in Figure 4 demonstrates the presence of the above elements along with carbon in the MNA-CSP-Pd-Cel sample. The XPS survey spectrum of MNA-CSP-Pd-Cel shows that the nanobiocatalyst surface contains the same elements as those identified by EDS. According to the XPS data (the data not shown), the binding energies of the Pd $3d_{5/2}$ for the MNA-CSP-Pd-Cel demonstrate two states with binding energies of 335.7 and 337.9 eV, which correspond two types of species: Pd(0) and Pd(+2).
The study of the biocatalyst by infrared Fourier spectroscopy showed that all the absorption bands inherent in magnetite and chitosan are present in the MNA-CSP spectrum. Two characteristic regions were identified in the Cel and MNA-CSP-Pd-Cel spectra: 852 cm^{-1} - characteristic band of the amino groups of the enzyme; 1,232 cm^{-1} – characteristic band involving the C-O-C bond, which is part of the protein molecules of the enzyme. These bands are absent in the MNA-CSP sample, which confirms the effective immobilization of cellulase on chitosan-coated magnetic nanoparticles.
The catalytic properties of immobilized cellulase (MNA-CSP-Cel) in enzymatic hydrolysis of CMC to glucose and palladium nanoparticles on the same support (MNA-CSP-Pd) during the hydrogenation of glucose to sorbitol were previously studied. The next step in our research was to create a bifunctional catalyst containing both palladium particles and immobilized cellulase MNA-CSP-Pd-Cel. The application was carried out sequentially: first, palladium nanoparticles were applied to magnetic particles modified with chitosan, after which cellulase immobilization was performed. The presence of palladium nanoparticles had no effect on the properties of immobilized cellulase. The activity, dependence on pH and temperature for the MNA-CSP-Pd-Cel sample were exactly the same as for the MNA-CSP-Cel sample.
Hydrolysis of CMC in the presence of immobilized cellulase is most effective at a temperature of 50-70 °C and pH 5-7. Glucose hydrogenation to sorbitol occurs at a temperature of 150 °C, pressure of (H2) 5 MPa, pH 7. And then it turns out that to carry out the “one-pot” chemoenzymatic cascade transformation CMC to sorbitol for our bifunctional nanobiocatalyst, we can use a maximum temperature of 70 °C and pH 7. In Table 1 The composition of the reaction mixture for 5, 10, and 15 h of CMC conversion to sorbitol is presented. In 5 h of the process, the full CMC conversion (enzymatic hydrolysis) has not yet been achieved. During the hydrolysis of CMC, as glucose is formed, the process of glucose hydrogenation to sorbitol proceeds in parallel. In 10 h, full CMC conversion was achieved, glucose conversion was 72.1 % and sorbitol selectivity was 97.1 %. In 15 h, complete glucose conversion was achieved, but a byproduct of mannitol was formed and the selectivity for sorbitol decreased to 80.4 %. This decrease in selectivity is probably due to the side effect of sorbitol isomerization into mannitol during prolonged exposure to the reactor.

Table 1: The composition of the reaction mixture at different time intervals

Time, h	Reaction mixture composition, %*			
	CMC	Glucose	Sorbitol	Mannitol
5	5.7	42.1	51.7	0.5
10	-	27.9	70.0	2.1
15	-	-	80.4	19.6

*Reaction conditions: volume of the reaction mixture 25 mL, the CMC concentration 0.25 % (w/v), the MNA-CSP-Pd-Cel loading 0.1 g, pH 7, temperature 70 °C, PH2 5 MPa, reaction time 15 h.

Experiments have been conducted on the reuse of the MNA-CSP-Pd-Cel catalyst to assess stability under optimized reaction conditions. After each cycle, the MNA-CSP-Pd-Cel catalyst was separated from the reaction mixture using a neodymium magnet, washed with a buffer solution (pH 7), and then reused. The catalytic efficiency of the bifunctional catalyst decreased after using it five times. CMC conversion and sorbitol yield decreased from 100 % to 90.8 % and from 80.4 % to 72.6 %, respectively. The result obtained: a sorbitol yield of 80.4 % could be obtained at 70 °C and 5 MPa H_2 for 15 h in the chemoenzymatic cascade transformation CMC to sorbitol in the presence of the bifunctional catalyst MNA-CSP-Pd-Cel was better than for the bifunctional catalyst Pd/Al_2O_3-MPA, which was used in the conversion of cellulose into sorbitol. A sorbitol yield of 78.5 % could be obtained at 180 °C and 5 MPa H_2 for 5 h.

3. Conclusions

A novel bifunctional nanobiocatalyst based on a functionalized magnetic support, MNA-CSP, and containing Pd NPs and immobilized Cel (MNA-CSP-Pd-Cel) was developed. This catalyst was designed for a one-pot chemoenzymatic cascade transformation of CMC to D-sorbitol which proceeds via two steps: enzymatic hydrolysis of CMC to D-glucose with immobilized Cel and hydrogenation of D-glucose to D-sorbitol with the Pd catalyst. The MNA-CSP-Pd-Cel nanobiocatalyst was synthesized with optimal concentrations of Cel and Pd and mild reaction conditions were selected for the cascade process. The result of this study was a successful

cascade reaction of CMC to produce D-sorbitol with a yield of 80.4 % D-sorbitol (at 70 °C, pH 7) and a hydrogen pressure of 5 MPa for 15 h, which significantly exceeds the published data for the same process. The complete characterization of MNA-CSP-Pd-Cel led us to understand the relationship between structure and properties and to realize that ultra-small Pd nanoparticles (1.0 nm) evenly distributed over the nanobiocatalyst are quite effective even at reaction temperatures below optimal. This work paves the way for the development of various bifunctional chemo-enzyme catalysts for single cascade transformations.

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