Postprint: Immobilization of 5-Hydroxymethylfurfural Oxidase in MOFs and Its Catalytic Activity

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Abstract

Immobilized enzymes, as green and highly efficient biocatalysts, exhibit significantly superior performance compared to free enzymes. Currently, the applicability of enzyme immobilization technology remains relatively limited, with research predominantly confined to model enzymes; thus, expanding the research scope of immobilized enzymes is of paramount importance. Metal-organic frameworks (MOFs) have been extensively investigated as carriers for enzyme immobilization in recent years; however, many characteristics of biologically functional enzyme-MOFs composites remain to be elucidated. In this study, a biomimetic mineralization synthesis approach was employed to immobilize 5-hydroxymethylfurfural oxidase (HMFO) into MOFs materials represented by zeolitic imidazolate framework-8 (ZIF-8), yielding a novel biocatalyst HMFO@ZIF-8. Scanning electron microscopy characterization revealed a morphology distinct from the classic rhombic dodecahedron. The Coomassie brilliant blue assay was utilized to determine protein concentration, and the calculated enzyme immobilization efficiency reached 89.0%. HMFO@ZIF-8 achieved a conversion rate of 84.3% for 5-hydroxymethylfurfural, with both yield and selectivity surpassing those of the free enzyme. This study expands the research scope of MOFs-immobilized enzymes and provides valuable insights for the investigation of biocatalysts based on other biomacromolecular composites.

Full Text

Immobilization of 5-Hydroxymethylfurfural Oxidase within MOFs for Catalysis

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Abstract

Immobilized enzymes represent green and efficient biocatalysts that typically exhibit superior performance compared to their free counterparts. However, current enzyme immobilization technologies remain limited in scope, with most research focusing on model enzymes. Expanding the range of enzymes studied in immobilization systems is therefore of significant importance. Metal-organic frameworks (MOFs) have attracted considerable attention as enzyme immobilization carriers in recent years, yet many characteristics of these functional enzyme-MOF composites remain to be fully explored. In this study, we employed a biomimetic mineralization synthesis method to immobilize 5-hydroxymethylfurfural oxidase (HMFO) within zeolitic imidazolate framework-8 (ZIF-8), a representative MOF material, yielding a novel biocatalyst designated HMFO@ZIF-8. Scanning electron microscopy revealed that its morphology differed from the classic rhombic dodecahedron structure typical of ZIF-8. Using the Coomassie brilliant blue method to determine protein concentration, we calculated an enzyme immobilization efficiency of 89.0%. The HMFO@ZIF-8 catalyst achieved an 84.3% conversion rate for 5-hydroxymethylfurfural, with both yield and selectivity exceeding those of the free enzyme. This work expands the research scope of MOF-immobilized enzymes and provides valuable insights for studying other biomacromolecular composite biocatalysts.

Keywords: MOFs; 5-hydroxymethylfurfural oxidase; immobilized enzyme; biocatalyst

Introduction

Enzymes possess unique characteristics including high selectivity, high catalytic activity, mild reaction conditions, and environmental friendliness, making them widely applicable in chemical engineering, pharmaceuticals, and food industries. However, free enzymes suffer from poor stability and difficult recovery, which severely limits their industrial applications. To address these limitations, enzyme immobilization technologies have emerged and continuously evolved. Traditional immobilization using carriers such as activated carbon, metal oxides, and clays can improve enzyme stability, but numerous problems persist, including low protein loading, enzyme leakage, disruption of molecular structure, and conformational changes. In response to these challenges, immobilization techniques based on novel carrier materials have gradually developed and been applied, with carriers evolving from traditional materials to new nanomaterials such as carbon nanotubes, graphene, metal-organic frameworks, and inorganic crystal composites. These novel carriers typically feature large surface areas, porous spatial structures, and substrate/product affinity, significantly improv-

ing immobilization efficiency and stability. Moreover, enzyme immobilization using these new composite materials has expanded application scope beyond biocatalysis to bioanalysis and biomedical engineering.

Among these novel carriers, metal-organic frameworks (MOFs) have attracted particular attention, with zeolitic imidazolate frameworks (ZIFs) synthesized via biomimetic mineralization becoming a research hotspot. ZIF-8 is a cage-structured material composed of organic and inorganic components that exhibits thermal and chemical stability, enabling successful applications in catalysis, separation, and gas adsorption. Its porosity facilitates the transport of small molecules, allowing selective interaction between biomacromolecules (such as enzymes) and the external environment. Additionally, ZIF-8 possesses a large surface area, well-defined porosity, and abundant functional groups. Most importantly, the synthesis of ZIF-8 occurs under mild conditions suitable for forming composites with biomolecules, demonstrating excellent biocompatibility that highlights its unique advantages as a carrier material. Researchers have extensively explored ZIF-8 for immobilizing various molecules including DNA, drugs, enzymes, and other proteins, with ZIF-8 immobilization significantly improving the thermal stability, organic solvent resistance, and storage lifetime of these biomacromolecules.

Current research on enzyme immobilization using novel materials, particularly ZIF-8, has primarily focused on model enzymes such as glucose oxidase, horseradish peroxidase, and lipase, resulting in a relatively narrow research scope. Since carrier selection must comprehensively consider the target enzyme's molecular size, amino acid composition, structural features, and physicochemical properties, no universal immobilization method exists for all enzyme classes, and broader studies on structure-property relationships and mechanisms remain lacking. Therefore, expanding the research scope of novel material-immobilized enzymes is crucial for fundamental and applied studies of enzyme-material composites. In 2015, Jin et al. demonstrated that ZIF-8 exhibited the highest adsorption capacity for 5-hydroxymethylfurfural (HMF) among various MOFs tested. 5-Hydroxymethylfurfural oxidase (HMFO), a glucose-methanol-choline oxidase, can effectively catalyze HMF conversion into high-value-added compounds. Given ZIF-8's specific adsorption properties for HMF, immobilizing HMFO within ZIF-8 should theoretically enhance catalytic performance. In the presence of cofactors, HMFO catalyzes HMF to produce 2,5-furandicarboxylic acid (FDCA), while in their absence, it primarily generates 2,5-diformylfuran (DFF) and 5-hydroxymethyl-furan-2-carboxylic acid (FFA). Since conventional ZIF-8 synthesis methods reported in literature involve organic solvents that can cause enzyme deactivation, this study employed a biomimetic mineralization method in aqueous solution to prepare ZIF-8 and achieve HMFO immobilization for the first time, yielding HMFO@ZIF-8 composites while investigating the effects of ZIF-8 synthesis components on HMFO activity. This work expands the research scope of ZIF-8-immobilized enzymes and provides insights for understanding biomimetic mineralization-based enzyme immobilization.

2.1 Instruments and Reagents

5-Hydroxymethylfurfural oxidase (molecular weight ~60 kDa) from *Pseudomonas nitroreducens* CICC 20703 was purchased from the China Center of Industrial Culture Collection and maintained by Research Group 1805 at the Dalian Institute of Chemical Physics, Chinese Academy of Sciences. Zinc acetate (Tianjin Damao Chemical Reagent Factory) and 2-methylimidazole (Sigma-Aldrich) were used, with all other unlisted reagents being analytical grade. HMF, DFF, and FFA standards (all chromatographic grade) were purchased from Sigma-Aldrich, InnoChem Science & Technology, and J&K Scientific, respectively. Equipment included an ML104 electronic balance (Mettler Toledo), Fresco21 micro refrigerated centrifuge (Beijing Chengmao Xingye Technology), BioTek microplate reader (BioTek Instruments), NULL HPLC system (Dalian Elite Analytical Instruments), JEOL SEM JSM-7800F scanning electron microscope (JEOL), and FV1000MPE laser confocal microscope (Olympus).

2.2.1 Synthesis of HMFO@ZIF-8 and Determination of Protein Concentration and Loading Rate

ZIF-8 is a MOF material with zeolitic imidazolate framework structure formed by reacting divalent metal salts with imidazole or its derivatives at room temperature. HMFO@ZIF-8 was synthesized via a one-pot method by adding HMFO during ZIF-8 formation. Briefly, 2.0 mL of 2-methylimidazole solution (160.0 mM) was mixed with 0.5 mL HMFO solution (1.0 mg/mL), followed by addition of 2.0 mL zinc acetate solution (40.0 mM). After stirring and overnight incubation, the resulting solid was washed three times with deionized water to obtain HMFO@ZIF-8. All supernatants were collected, and protein concentration was determined using the Coomassie brilliant blue staining method with bovine serum albumin (BSA) as the standard to calculate HMFO loading rate.

BSA standard solutions (0–0.1 mg/mL) were prepared, and 50 L of each sample protein and BSA standard were added to a 96-well plate. After adding 200 L Coomassie brilliant blue reagent to each well and reacting for 3 minutes, absorbance at 595 nm was measured. Protein concentration was calculated from the BSA standard curve, and HMFO loading rate was determined using Equation (1), where A represents the amount of protein added (mg) and B represents the amount of protein in the supernatant (mg).

2.2.2 SDS-PAGE Characterization of HMFO@ZIF-8

To avoid interference from acetic acid in SDS-PAGE analysis, HMFO@ZIF-8 was dissolved in appropriate glacial acetic acid, and proteins were precipitated using a 1:1 ethanol-acetone mixture (4:1 volume ratio) to remove excess organic ligands, metal ions, and acetic acid solution. After storage at -20°C for 2 hours and centrifugation, the supernatant was discarded, and the precipitate was washed three times with absolute ethanol. The pellet was dissolved in 2.0%

SDS with sonication, and 40 L protein solution was mixed with 10 L 5× sample buffer, boiled for 10 minutes, and analyzed by SDS-PAGE. Supernatant from the immobilization process and free HMFO were similarly analyzed for comparison.

2.2.3 Confocal Microscopy Characterization of HMFO@ZIF-8

HMFO (1.0 mg/mL) was dissolved in 10.0 mL PBS buffer (20.0 mM, pH 7.9) containing 3.0 mg fluorescein isothiocyanate isomer (FITC) and stirred at room temperature for 24 hours. After dialysis for 3 days and lyophilization, FITC-labeled HMFO@ZIF-8 particles were prepared using the method described in Section 2.2.1 under dark conditions. Fluorescence detection was performed using confocal microscopy with FITC green fluorescence channel (excitation wavelength 488 nm, emission wavelength 500–550 nm).

2.2.4 Scanning Electron Microscopy Characterization of HMFO@ZIF-8

Prepared HMFO@ZIF-8 samples were lyophilized, and small amounts were placed in the scanning electron microscope for morphological characterization at 1.0 kV acceleration voltage, 8.0 mm working distance, and 5000–10000× magnification.

2.2.5 Enzyme Activity Assay

As a flavin-dependent oxidase, HMFO oxidizes aromatic vanillyl alcohol to vanillin, which exhibits strong UV absorption at 320 nm. For activity measurement, 50 L HMFO solution (3.0 mg/mL) was added to 1.0 mg/mL vanillyl alcohol in potassium phosphate buffer (50.0 mM, pH 8.0) and incubated at 25°C for 1 hour with shaking. After reaction, samples were centrifuged at 12,000 rpm for 10 minutes (free enzyme solution was boiled for 10 minutes before centrifugation), and OD values at 320 nm were measured to calculate enzyme activity. One unit of enzyme activity (U) was defined as the amount of enzyme catalyzing the formation of 1 mol product per minute.

2.2.6 Catalytic Oxidation of HMF by HMFO@ZIF-8

The catalytic performance of HMFO@ZIF-8 was evaluated in both aqueous and sodium phosphate buffer (20.0 mM, pH 7.9) systems. Reactions were initiated by adding the entire amount of HMFO@ZIF-8 prepared from a 4.0 mL system to 0.5 mg/mL HMF solution. An air-filled balloon was connected to the round-bottom flask to ensure adequate oxygen supply, with the balloon refilled every 12 hours to maintain inflation. Reactions proceeded with magnetic stirring at 30°C for 85 hours, with free HMFO as control. After completion, HMFO@ZIF-8 was dissolved with appropriate hydrochloric acid, centrifuged at 12,000 rpm for 10 minutes, filtered through a 0.22 m aqueous membrane, and analyzed by HPLC. HMF conversion was calculated using Equation (2), while DFF and FFA yields

were calculated using Equation (3), where concentrations were determined from standard curves.

HPLC analysis conditions: Hi-PlexH column (300 mm × 7.7 mm, Agilent), column temperature 60°C, UV-230 UV-Vis detector, mobile phase of 5 mM sulfuric acid at 0.7 mL/min flow rate, and detection wavelength of 268 nm.

3.1 Synthesis and Loading Capacity of HMFO@ZIF-8

Enzyme immobilization must consider the target enzyme's properties, including immobilization method, immobilization rate, enzyme activity, and stability. This study employed biomimetic mineralization to prepare HMFO@ZIF-8 composites for the first time using a non-model enzyme, achieving a loading capacity of 89% (Figure 1 [Figure 1: see original paper]), which far exceeds traditional carriers. For comparison, Xing et al. reported only 32.2% immobilization efficiency for CotA laccase on calcium alginate, while Cherian et al. achieved a maximum of 75% efficiency for cellulase immobilization on MnO_2 nanoparticles. This simple, mild synthesis method not only provides highly efficient protein encapsulation but also avoids the lengthy and cumbersome procedures associated with graphene oxide and carbon nanotube materials. Compared with two-dimensional graphene oxide, the three-dimensional MOF-based immobilization approach can create tailored pore sizes for protein molecules, enhancing enzyme-substrate binding efficiency while preventing degradation by other macromolecules.

3.2 SDS-PAGE Analysis of HMFO@ZIF-8

To prevent interference from glacial acetic acid in SDS-PAGE analysis, proteins were precipitated with organic solvents to remove excess organic ligands, metal ions, and acetic acid solution. SDS-PAGE analysis revealed a protein band in the HMFO@ZIF-8 powder, while no band was detected in the supernatant (Figure 2 [Figure 2: see original paper]), indicating that HMFO was essentially completely loaded into ZIF-8, consistent with the loading efficiency results.

3.3 Confocal Microscopy Characterization of HMFO@ZIF-8

Using FITC as a fluorescent probe, HMFO was labeled and HMFO-FITC@ZIF-8 was prepared. Confocal microscopy revealed uniformly distributed fluorescent spots (Figure 3 [Figure 3: see original paper]). Since neither 2-methylimidazole nor zinc acetate exhibits fluorescence, the fluorescent signal must originate from FITC-labeled HMFO, further confirming successful enzyme loading into ZIF-8.

3.4 Scanning Electron Microscopy Characterization of HMFO@ZIF-8

Lyophilized HMFO@ZIF-8 samples were observed by scanning electron microscopy, which revealed a nanoflower structure rather than the classic rhombic dodecahedron morphology (Figure 4a). This suggests that protein incorporation altered the crystallization process of ZIF-8. Previous studies have shown that enzyme@ZIF-8 composite morphology varies depending on the protein molecule, with BSA encapsulation yielding the classic rhombic dodecahedron under identical conditions, while ovalbumin, ribonuclease, glucose dehydrogenase, lipase, lysozyme, insulin, horseradish peroxidase, trypsin, and urease each produced different morphologies. To verify this hypothesis, BSA@ZIF-8 was prepared as a control, yielding the expected rhombic dodecahedron structure (Figure 4b [Figure 4: see original paper]), confirming our speculation.

3.5 Catalytic Conversion of 5-Hydroxymethylfurfural by HMFO@ZIF-8

Using vanillyl alcohol as substrate, the immobilized enzyme retained 20% of its original activity. Investigation of different concentrations of 2-methylimidazole and zinc acetate revealed that both components reduced HMFO activity to varying degrees (Figure 5 [Figure 5: see original paper] and Figure 6 [Figure 6: see original paper]), likely contributing to the observed activity loss after immobilization. Despite numerous reported immobilization materials, no single material is universally suitable for all enzymes, as solution pH, metal ions, and temperature all affect enzyme activity. Zinc ions inhibited HMFO activity in a concentration-dependent manner, while 2-methylimidazole solution is strongly alkaline, reducing HMFO activity to approximately 40% at concentrations above 0.1 M, possibly due to structural changes causing bond cleavage and subunit dissociation. These findings provide valuable insights for future ZIF-8 immobilization studies.

HMF is an important biomass-derived platform compound containing a furan ring, aldehyde, and hydroxymethyl groups. HMFO selectively oxidizes HMF to produce C6 bulk chemicals including DFF, FFA, and FDCA, which have applications in pharmaceuticals, plastics, and fuels. In the absence of cofactors, HMFO converts HMF to DFF and FFA. HMFO@ZIF-8 was used to catalyze HMF in different reaction systems, and after 85 hours, HPLC analysis was performed (Figure 7 [Figure 7: see original paper]). Standard curves for HMF, DFF, and FFA were established by linear fitting of peak area versus concentration (Figure 8 [Figure 8: see original paper]), enabling quantification of reactants and products. The results showed 84.3% HMF conversion, 64.1% DFF yield, and 17.7% FFA yield, with a combined yield of 81.8% and selectivity of 97%—both significantly higher than the 71.7% achieved with free enzyme (Table 1). Although HMFO@ZIF-8 showed lower activity than free enzyme when using vanillyl alcohol as substrate, its selectivity and yield for HMF oxidation were

superior. Despite the altered ZIF-8 structure upon HMFO incorporation, the material retained porosity that provided specific adsorption for HMF, enhancing the binding rate between immobilized enzyme and substrate and consequently accelerating catalytic efficiency.

Conclusion

Under mild and simple conditions, we successfully immobilized the non-model enzyme HMFO using biomimetic mineralization, obtaining catalytically active HMFO@ZIF-8 with 89.0% enzyme loading. Morphological characterization revealed a nanoflower structure distinct from the classic rhombic dodecahedron. Activity assays showed the immobilized enzyme retained 20% of its original activity, with deactivation likely caused by the strong alkalinity of 2-methylimidazole and high concentrations of zinc acetate. Due to ZIF-8's specific adsorption of HMF, HMFO@ZIF-8 achieved 84.3% HMF conversion with a combined DFF and FFA yield of 81.8% and 97% selectivity—substantially higher than the 71.7% obtained with free enzyme. This study demonstrates that biomimetic mineralization strategies for immobilizing biomacromolecules must carefully consider the relationship between target enzyme structural properties and the immobilization carrier to maximize enzyme performance. Our findings provide valuable insights for expanding fundamental and applied research on enzyme@ZIF-8 composites.

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