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Synthesis of Metal-Organic Framework with Fe and Mo as central metals and Its Biological Effects on *Azotobacter vinelandii*

Hua Chen

Abstract*—This study successfully synthesized metal-organic framework with Fe and Mo as central metals (FeMo-MOF), with XRD, SEM and XPS characterizations confirming its bipyramidal morphology and expected Mo/Fe coordination structure. At concentrations of 20-60 mg/L, colony forming units (CFU) doubled, with the fastest logarithmic growth rate observed at 20 mg/L. However, higher concentrations (80-100 mg/L) limited CFU growth due to physical damage caused by the material's sharp edges. The 20 mg/L treatment group showed a 20% increase in nitrogenase activity, while ^{15}N isotope analysis indicated a slight (statistically insignificant) improvement in total nitrogen fixation, attributed to Mo released from the MOF acting as a nitrogenase cofactor. Although FeMo-MOF did not alter GSH levels (indicating unactivated antioxidant systems), elevated MDA content demonstrated that the material's sharp structure induced membrane lipid peroxidation damage. This research provides important insights for environmentally friendly MOF design: balancing metal selection (prioritizing non-toxic metals) with morphology optimization (reducing physical damage) to achieve efficient and safe agricultural applications.

***Index Terms*—Metal-organic framework; Metal element; nitrogen fixation; Oxidative stress; Environmental effects**

INTRODUCTION

Biological nitrogen fixation can be traced back approximately 4 billion years, covering almost the entire history of life on Earth and occurring nearly simultaneously with photosynthesis [1]. Nitrogen fixation is the process by which molecular nitrogen is reduced to ammonia or other nitrogen-containing compounds. Natural nitrogen fixation can be categorized into abiotic and biological nitrogen fixation. Despite significant achievements in artificial nitrogen fixation, over 80% of global nitrogen fixation still relies on biological nitrogen fixation, where nitrogen-fixing microorganisms produce nitrogenase to catalyze the reduction of nitrogen gas to ammonia. Nitrogen exists in various oxidation states, and microbial activity facilitates the conversion between these forms, constituting the global biogeochemical nitrogen cycle [2].

Free-living nitrogen-fixing bacteria are certain bacteria capable of independently fixing nitrogen, converting molecular nitrogen into ammonia, which is then synthesized into amino acids and proteins. These include aerobic genera such as *Azotobacter* and *Azospirillum*, facultative anaerobes like *Klebsiella*, and some anaerobic species of *Clostridium*. They are often used as model strains for research. Symbiotic nitrogen-fixing bacteria can only fix nitrogen or do so efficiently when in symbiosis with plants, exhibiting nitrogen-fixing efficiency dozens of times higher than free-living systems.

Hua Chen, College of Chemical and Environment, Southwest Minzu University, Chengdu, China

Examples include the root nodule symbiosis formed by Rhizobium with legumes, the symbiosis of *Bradyrhizobium* with elm family plants, the root nodule symbiosis formed by *Frankia* with non-leguminous plants like *Casuarina* and *Alnus*, and symbioses formed by certain cyanobacteria with plants. Associative nitrogen-fixing bacteria represent an intermediate type between free-living and symbiotic nitrogen-fixing bacteria. They do not form root nodules but live in the rhizosphere of certain plants to fix nitrogen, displaying strong specificity [3].

The "ultimate executor" of nitrogen fixation is the nitrogenase within nitrogen-fixing microorganisms [4]. Nitrogenases can be broadly divided into three types: the molybdenum-iron nitrogenase (Mo-Nitrogenase), found in all nitrogen-fixing organisms and the only one known to form symbioses with higher plants; and two alternative nitrogenase systems, the vanadium-iron nitrogenase (V-Nitrogenase) and the iron-only nitrogenase (Fe-only Nitrogenase) [5-6]. These nitrogenases all contain a smaller component protein called the Fe protein, which includes a [4Fe-4S] cluster and a MgATP-dependent electron transfer protein. The larger component, known as dinitrogenase, contains the active-site metal cluster. Nitrogenase-mediated nitrogen fixation involves three basic electron transfer steps: first, the reduction of the Fe protein via electron carriers such as ferredoxin or flavodoxin; second, the transfer of a single electron from the Fe protein to the MoFe protein, coupled with MgATP hydrolysis for each electron transfer; and third, the transfer of electrons and protons to the substrate, binding with the MoFe cofactor of the MoFe protein. All known nitrogenases require at least two electrons to reduce the substrate fully, necessitating repeated cycles of steps one and two until sufficient electrons are provided for complete substrate reduction [6]. Each electron transfer between the Fe protein and the MoFe protein involves a mandatory association or dissociation cycle of the protein complex, with the dissociation step identified as the rate-limiting factor for the overall reaction [7-8].

Nitrogen loss refers to the process by which nitrogen escapes from the soil into water bodies or the atmosphere. In agricultural fields, nitrogen typically exists in the forms of nitrate and ammonium, which are easily washed away by water or leached, or converted into gaseous nitrogen (N_2) through soil nitrification and denitrification processes, volatilizing into the atmosphere. Nitrogen loss is a major environmental challenge in agriculture, as it can lead to water eutrophication, groundwater pollution, and increased greenhouse gas emissions [9]. Nitrogen-fixing bacteria utilize nitrogenase to reduce nitrogen (N_2) to ammonium, but nitrogenase must acquire essential metals such as molybdenum (Mo) and iron (Fe) for its metal cofactors. Research shows that synthesizing molybdenum-iron

compounds positively influences nitrogenase activity [10]. Loading molybdenum and iron as metal active centers onto MOF frameworks and releasing them into the environment can achieve a slow-release effect of metal ions. Therefore, this study attempts to synthesize Fe/Mo-MOF materials to sustainably enhance the nitrogen-fixing activity of nitrogen-fixing bacteria and address nitrogen loss issues.

Metal-organic frameworks (MOFs) are a class of materials formed by the self-assembly of organic ligands and metal centers, featuring adjustable pore sizes. Compared to traditional inorganic porous materials, MOFs exhibit larger specific surface areas, higher porosity, and more diverse structures and functions, making them widely applicable in gas adsorption and separation, sensors, drug delivery, and catalytic reactions [11]. MOFs were first successfully synthesized in the 1960s by the Yaghi group in the U.S. and the Kitagawa group in Japan. Since then, a variety of MOF materials have been developed, characterized by their diversity, functionality, large specific surface area, tunable pore size, biomimetic catalysis, and biocompatibility [12]. These porous coordination polymers form infinite networks with backbones consisting of metal ions as linkers, creating inorganic-organic hybrid polymers, also known as porous metal-organic frameworks. The coordination between metals and organic compounds forms specific framework structures, combining the activity of metals with the properties of organic ligands, functional group selectivity, and other physicochemical characteristics, as well as the unique spatial structures resulting from coordination [13].

In recent years, among the numerous metal-organic frameworks, MOFs with unsaturated metal sites have shown excellent potential in adsorption and catalysis, attracting widespread attention [14]. For instance, the MOF-74 structure synthesized by Yaghi et al. features one-dimensional channels and unsaturated metal sites, constructed from straight-chain organic ligands of varying lengths and metal clusters. To date, nine MOF-74 series materials with different pore diameters have been developed, combining microporous and mesoporous structures within a single topology [15]. Moreover, MOF-74 series materials synthesized from different metal sources have demonstrated outstanding performance in gas adsorption, becoming a focal point in MOF application research [12].

With the widespread use of metal-organic frameworks (MOFs), they inevitably enter the environment, posing potential threats to various organisms. In 2022, Ouyang et al. conducted the first study on the effects of the iron-based metal-organic framework MIL-53(Fe) on the growth and activity of *Azotobacter vinelandii*. MIL-53(Fe) inhibited bacterial growth at relatively low concentrations (50 mg/L) and nearly completely suppressed it at 140 mg/L. The results indicated that MIL-53 remains stable in aqueous systems, and its toxicity stems from mechanical damage to the cell wall of *Azotobacter vinelandii* caused by MIL-53 [16].

In previous studies, soil nitrogen-fixing bacteria strictly regulate their metal uptake to acquire essential metals (such as Fe, Mo, and V for nitrogenase cofactors) while excluding toxic metals. Kraepiel et al. proposed that the catechol compounds they release, identified as siderophores of nitrogen-fixing bacteria, can bind various metals besides iron. At low concentrations, essential metals (Fe, Mo, V) bound to siderophores are absorbed by bacteria via specialized transport systems [17]. In topsoil (leaf litter), metals are primarily bound to plant-derived organic matter, and siderophores extract essential metals from natural ligands, delivering them to bacteria. This process appears to be a key component of the mutualistic relationship between trees and soil diazotrophs: trees produce leaf litter, providing nitrogen-fixing bacteria with an organic-rich, micronutrient-rich environment, while the bacteria in turn supply the ecosystem with new nitrogen [18].

In summary, research indicates that synthesizing molybdenum-iron compounds positively influences the nitrogen-fixing activity of nitrogen-fixing bacteria. Loading molybdenum and iron as metal active centers onto MOF frameworks and releasing them into the environment can achieve a slow-release effect of metal ions that benefit nitrogen-fixing bacteria. Therefore, this study attempts to synthesize FeMo-MOF materials to enhance biological nitrogen fixation activity. The impact of MOF-74(Mg) on the photosynthesis was indicated by the chlorophyll content, photosynthetic rate, and chlorophyll fluorescence parameters. The metal content changes and oxidative stress were also recorded. The implications to the applications and the design of safe MOF materials are discussed.

EXPERIMENTAL

Materials preparation

$FeCl_3$ and $MoCl_5$ were mixed at a specific ratio (total amount: 10 mmol) and combined with 10 mmol of 1,4-benzenedicarboxylic acid (H_2BDC) in 40 mL of N, N-dimethylformamide (DMF). The reaction mixture was then transferred to a Teflon-lined stainless-steel autoclave and heated at 150 °C for 15 h. After cooling to room temperature, the solid product was collected, washed with deionized water under stirring, and filtered. The resulting residue was dried overnight in an oven at 60 °C to obtain FeMo-MOF. The synthesized FeMo-MOF was characterized by X-ray photoelectron spectroscopy (XPS, ESCALAB 250XI, Thermo Fisher Scientific, USA), scanning electron microscopy (SEM, JSM-7500, JEOL, Japan), and X-ray diffraction (XRD, XD-6, Purkinje General Instrument Co., China).

Growth assay

For growth curves, *A. vinelandii* was inoculated in 50 mL culture medium in the presence/absence of FeMo-MOF at concentrations of 0-100 mg/L. Because FeMo-MOF did not disperse well in culture medium, the samples were shaken during adding and incubation. The optical density of the culture system was recorded at 600 nm (OD_{600}) with time intervals of 12 h for each flask. For CFU counts, 50 μL of the aforementioned bacterium suspension was collected at 72 h postexposure. The suspension was inoculated on solid medium after serial dilution, and CFU was counted after

another 48 h by colony counter (Scan-300, Wisdom Shanghai Instrument Co., China).

Nitrogen fixation activity assay of A. vinelandii

The absolute nitrogenase activity of *A. vinelandii* was determined by the acetylene reduction assay. A 10 mL aliquot of bacterial culture co-cultured with FeMo-MOF for 3 days was injected into a headspace vial, which was then sealed and evacuated. Subsequently, 12 mL of gas mixture (Ar: O_2: C_2H_2 = 6: 1: 1) was introduced, and the vial was incubated under the same culture conditions for 2 hours before being boiled in a water bath to inactivate the bacteria. Ethylene content was measured by gas chromatography (GC9720, Zhejiang Fuli Analytical Instrument Co., China). Nitrogenase activity was expressed as acetylene reduction activity (ARA) per unit protein. The relative nitrogen fixation rate of *A. vinelandii* was determined by the ^{15}N isotope dilution method. To 1 L of liquid culture medium, 0.1 g of ^{15}N-KNO_3 (Adamas Co., China) and 0.4 g of regular KNO_3 were added. After 3 days of co-culture with FeMo-MOF, 1 mL of bacterial suspension was collected, pre-frozen, and lyophilized. Exactly 1 mg of each sample was weighed, wrapped in tin foil, and degassed. The $^{15}N/^{14}N$ ratio was determined using isotope ratio mass spectrometry (IRMS, ISOPRIME VISION, Elementar, Germany).

Oxidative Stress Assay of A. vinelandii

Following 3-day exposure to FeMo-MOF, *A. vinelandii* cultures were collected with three replicates per treatment. For each sample, 10 mL of culture medium was centrifuged at 6000 rpm for 10 minutes. The cell pellet was resuspended in 1 mL of sterile ultrapure water and homogenized. Cellular disruption was then performed using a tissue grinder. The concentrations of glutathione (GSH) and malondialdehyde (MDA) were subsequently determined according to the manufacturer's protocols (Nanjing Jiancheng Bioengineering Institute, China).

RESULTS AND DISCUSSION

Characterization of FeMo-MOF Materials

FeMo-MOF was synthesized via hydrothermal reaction in DMF. The as-prepared FeMo-MOF appeared as pure-phase light orange crystalline powder. The typical XRD pattern of FeMo-MOF (Fig. 1a) displayed characteristic peaks at 10° and 12°, consistent with previous reports. SEM observations revealed that the FeMo-MOF particles exhibited bipyramidal morphology (Fig. 1b) with an average size of approximately 1.6 μm. Larger particles demonstrated better-developed and more elegant crystalline shapes.

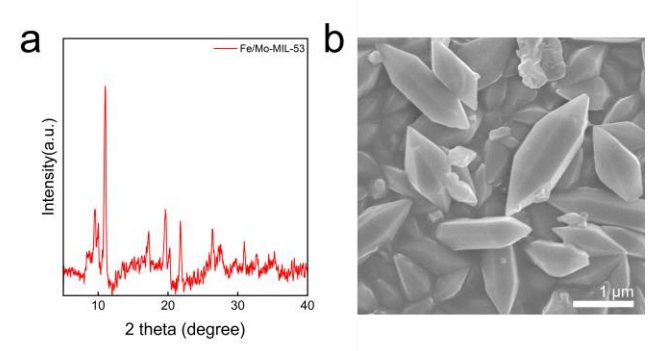

Figure 1 Material characterization of FeMo-MOF: (a) XRD; (b) SEM.

XPS analysis showed distinct peaks at 235.7 eV and 232.5 eV corresponding to Mo 3d orbital electrons (Fig. 2a), while peaks at 725.2 eV and 711.8 eV were attributed to Fe 2p orbital electrons (Fig. 2b). These spectroscopic data confirmed the successful incorporation of Mo into the FeMo-MOF framework, verifying the successful synthesis of FeMo-MOF and its readiness for subsequent experiments.

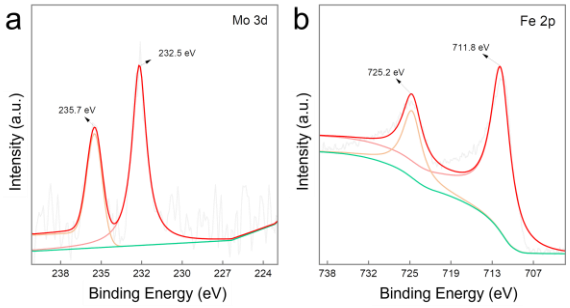

Figure 2 XPS characterization of FeMo-MOF: (a) Mo 3d; (b) Fe 2p.

Effect of FeMo-MOF on A. vinelandii Growth

The addition of FeMo-MOF significantly enhanced the growth of nitrogen-fixing bacteria. Growth curve analysis revealed that all treatment groups exhibited higher optical density (OD) values compared to the control group. Notably, the 20 mg/L treatment demonstrated the fastest growth rate during the logarithmic phase. By 72 hours, the OD values of all treatment groups plateaued, reaching their growth peaks (Fig. 3a). Colony forming unit (CFU) counts further corroborated these findings (Fig. 3b). FeMo-MOF concentrations between 20-60 mg/L substantially promoted bacterial growth, with the first three treatment groups more than doubling the viable cell counts. However, at higher concentrations (80-100 mg/L), while OD values continued to increase, CFU counts showed limited improvement. This discrepancy is attributed to physical damage caused by the sharp edges and angular morphology of FeMo-MOF particles during shake-flask cultivation, despite the apparent biomass increase.

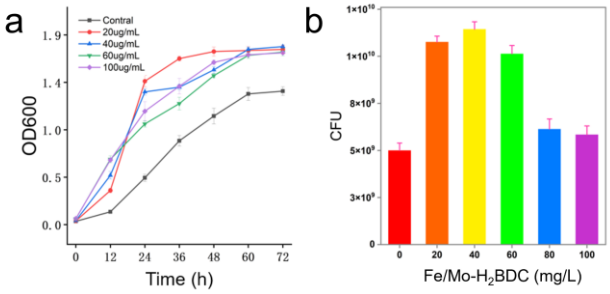

Figure 3 Effect of FeMo-MOF on *A. vinelandii* growth: (a) Growth curve; (b) Colony counting (CFU).

Impact of FeMo-MOF on Nitrogen Fixation Activity

FeMo-MOF significantly enhanced the nitrogenase activity of diazotrophic bacteria. Functioning as an enzyme-mimicking MOF, it releases both Mo and Fe ions into the culture system. Notably, molybdenum - an essential metal cofactor for nitrogenase - particularly contributed to

the observed activity enhancement when supplemented through FeMo-MOF. Experimental results demonstrated optimal nitrogenase stimulation at 20 mg/L FeMo-MOF concentration, showing approximately 20% higher activity compared to the control group (Fig. 4a). All treatment groups exhibited superior nitrogen fixation activity relative to the control. The ^{15}N isotope dilution assay revealed that FeMo-MOF addition marginally increased total nitrogen fixation (Fig. 4b), though this enhancement did not reach statistical significance. This suggests that while FeMo-MOF effectively boosts nitrogenase activity, its impact on overall nitrogen assimilation remains limited under the tested conditions.

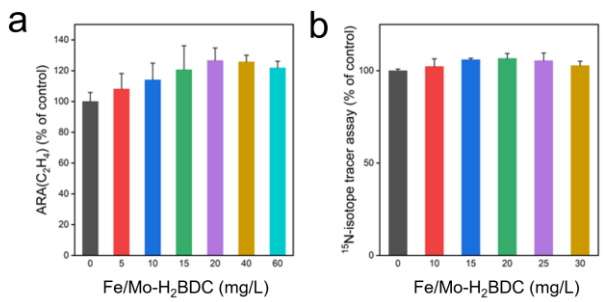

Figure 4 Effect of FeMo-MOF on nitrogen fixation activity of *A. vinelandii*: (a) Acetylene reduction assay (ARA); (b) ^{15}N isotope tracer assay.

Oxidative Stress Response of A. vinelandii to FeMo-MOF

As the primary driver of biological nitrogen fixation, the redox homeostasis and energy metabolism stability of nitrogen-fixing bacteria directly determine soil nitrogen supply capacity. Recent studies have revealed that MOFs can induce cellular metabolic collapse through multiple pathways, including interference with reactive oxygen species (ROS) scavenging systems and disruption of ion gradient homeostasis. Glutathione (GSH), a tripeptide composed of glutamate, cysteine and glycine, plays crucial physiological roles in antioxidant defense, detoxification, immune enhancement, and regulation of various biochemical reactions. Notably, GSH levels remained stable following FeMo-MOF exposure (Fig. 5a), indicating the material did not activate the bacterial antioxidant defense system. Malondialdehyde (MDA), a terminal product of lipid peroxidation, serves as a reliable biomarker for evaluating oxidative damage to cellular membranes. This peroxidation process is typically initiated by free radicals, particularly under aerobic conditions where radicals attack membrane lipids, especially polyunsaturated fatty acid-rich phospholipids. The observed MDA elevation (Fig. 5b) suggests that FeMo-MOF's sharp-edged morphology caused physical membrane damage during cultivation, subsequently triggering lipid peroxidation cascades in *A. vinelandii* cells. This membrane compromise may potentially affect cellular physiological activities while paradoxically not eliciting antioxidant responses.

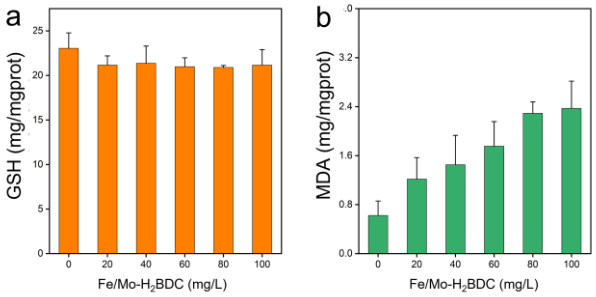

Figure 5 Oxidative stress of *A. vinelandii* induced by FeMo-MOF: (a) GSH; (b) MDA.

CONCLUSION

In summary, this study synthesized FeMo-MOF for the first time, and the characterization results confirmed the successful preparation of this material, while also testing its biological effects on nitrogen-fixing bacteria. The test results showed that the addition of FeMo-MOF greatly promoted the growth of nitrogen-fixing bacteria, with the most significant promotion observed at 20-60 mg/L, where CFU increased by more than double. At the same time, the addition of FeMo-MOF stimulated nitrogenase activity, with the highest promotion at 20 mg/L, increasing by about 20%, and there was also a slight improvement in total nitrogen fixation. Finally, we measured the oxidative stress of nitrogen-fixing bacteria. Due to the sharp surface of FeMo-MOF, it caused damage to cell membranes, resulting in increased MDA content. Our results indicate that the selection of metal elements should be fully considered when designing MOF materials to achieve environmental benefits. Non-toxic metals should be preferred to obtain similar framework structures with higher safety. We hope our research will contribute to the environmental safety assessment and safe application of MOF materials.

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