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Synthesis of novel laccase-biotitania biocatalysts for malachite green decolorization

Xinying Zhang,[1,2] Meiyin Wang,[1] Linlin Lin,[1] Gao Xiao,[1] Zhenping Tang,[1] and Xuefeng Zhu[3,4,*]

College of Environment and Resources, Fuzhou University, Fuzhou, Fujian 350108, PR China,[1] Research Institute of Photocatalysis, State Key Laboratory of Photocatalysis on Energy and Environment, Fuzhou University, Fuzhou 350002, China,[2] Shanghai Key Lab for Urban Ecological Processes and Eco-Restoration, School of Ecological and Environmental Sciences, East China Normal University, 500 Dongchuan Rd., Shanghai 200241, PR China,[3] and Section Sanitary Engineering, Department of Water Management, Faculty of Civil Engineering and GeoSciences, Delft University of Technology, 2628CN Delft, The Netherlands[4]

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Biomimetic mineralization has emerged as a novel tool for generating excellent supports for enzyme stabilization. In this work, protamine was used to induce titanium (IV) bis(ammonium lactato) dihydroxide (Ti-BALDH) into titania nanoparticles. This biomimetic titanification process was adopted for laccase immobilization. Laccase-biotitania biocatalyst was prepared and the effect of different parameters (buffer solution, titania precursor concentration, protamine concentration, and enzyme loading) on the encapsulation efficiency and recovery of laccase were evaluated. Compared with free laccase, the thermal and pH stability of immobilized laccase were improved significantly. In addition, laccase loaded on titania was effective at enhancing its storage stability. After seven consecutive cycles, the immobilized laccase still retained 51% of its original activity. Finally, laccase-biotitania biocatalysts showed good performance on decolorization of malachite green (MG), which can be attributed to an adsorption and degradation effect. The intermediates of the MG degradation were identified by gas chromatography-mass spectrometry (GC–MS) analysis, and the most probable degradation pathway was proposed. This study provides deeper understanding of the laccase-biotitania particles as a fast biocatalyst for MG decolorization.

[**Key words:** Laccase; Enzyme immobilization; Biomimetic titania; Malachite green; Wastewater treatment]

The application of industrial dyes in the fields of paints, cosmetics, clothes, and foods are accompanied by various risks on human health (1). Processes for the removal of dyes have attracted increasing attention from health professionals and environmentalists (2). The extensive use of malachite green (MG), in particular, has been shown to harm humans and animals, causing various types of cancer, and affecting the respiratory and reproductive systems (e.g., leading to reduced fertility (3,4)). MG is a triphenylmethane dye, highly soluble in water. The removal of MG can be done by chemical methods or biological treatments. Chemical methods, including fenton oxidation and photoelectrocatalysis among others, have several disadvantages of high costs and secondary pollutant generation (5). Biological processes, however, entail low running costs and marginal environmental risks. Among them, enzymes have emerged as a particularly effective process (6).

Laccases (EC1.10.3.2; oxygen oxidoreductases and polyphenol oxidases containing copper) have gained attention in the field of dye removal due to their nontoxicity and high specificity in biotechnology and biochemical research (7,8); they are environmentally friendly biocatalysts. They are also highly efficient and their high enzymatic activity has been widely applied in many processes in biotechnology (9). In a recent investigation, laccases showed great potential for decolorizing an extensive range of industrial dye wastewater, especially azo dyes and triphenylmethane dyes (MG being among them) (10,11). The limitations for the use of free laccases in these processes are their low operational stability and poor reusability. However, immobilized enzyme techniques can enhance laccases' thermal and chemical stability, making it simple to recover and reuse them.

A number of studies have shown that laccases can be successfully immobilized on a variety of matrices via physical adsorption, covalent bonding, cross-linking, and entrapment (12). However, these conventional immobilization methods have limitations, such as activity losses during immobilization and mass-transfer restrictions (13). To address these issues, the use of nanoparticles as carrier materials that immobilize laccase have attracted growing levels of attention (14). In a previous study, laccase was encapsulated into chitosan nanoparticles and received high enzyme immobilization efficiency (15). The stability of the nano-encapsulated laccase against microbial degradation in soil, compost, and wastewater was significantly increased, while the free laccase was almost completely inactivated. Ji et al. (16) also pointed out that laccase could be immobilized onto the functionalized TiO_2 nanoparticle surface. These biocatalytic nanoparticles exhibited good performance for the treatment of bisphenol-A and carbamazepine. Nanoparticles not only provide a higher specific surface area for laccase attachment but can also allow a higher degree of freedom for the enzyme active

* Corresponding author at: School of Ecological and Environmental Sciences, East China Normal University, 500 Dongchuan Rd., Shanghai 200241, PR China. Tel./fax: +86 21 50218524.
E-mail address: xfzhu@sspu.edu.cn (X. Zhu).

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sites as they minimize the lateral interaction between enzymes (17–19).

An emerging approach for enzyme immobilization is entrapment in nanostructure biocatalysts, a process called biomimetic mineralization (14). Biomimetic mineralization refers to the nucleation and growth of inorganic minerals controlled by biomolecules under normal reaction conditions like neutral pH, rapid reaction rate, and controllable morphology (20). Several biomineral materials used in the field of enzyme immobilization have been studied (21). Precipitation of inorganic minerals (silica, zirconia, and titania) can be induced by various factors (e.g., R5 peptide, lysozyme, protamine, branched polyethyleneimine) (22,23). Materials based on titania have specific physical and chemical properties that make titania-based materials more suitable for enzyme immobilization at a large scale, compared, for instance, with silica-based materials (24). The immobilization of certain enzymes on titania via biomimetic titanification process has been reported. Ren et al. (25) found that polyethylenimine as coating and template induced the hydrolysis and condensation of the titanium precursor to form titania, and achieved stable amine dehydrogenase immobilization with a high entrapment efficiency up to 90%. Jiang et al. (26) reported that horseradish peroxidase could be immobilized in phospholipid-templated titania particles induced by dodecylamine through a biomimetic process. The result showed that the stability and tolerance capacity of immobilized horseradish peroxidase were both enhanced; and the immobilized enzyme presented a high efficiency in removal of phenolics and dye. However, to date there has been no report describing the bioinspired formation of titania induced by protamine for laccase immobilization. It was found that protamine displayed a good titania-precipitating ability. The antibacterial and pharmaceutical properties of protamine are outstanding. In addition, the relatively low price, facile purification and easy availability from fish, avian and mammalian sperm nuclei ensure protamine is a promising inducer for titania-precipitating (27).

In this paper, laccases are immobilized on titanium oxide via biomineral processes (Fig. 1). The morphological and functional characteristics of biotitania particles were characterized; the factors influencing laccase immobilization efficiency were evaluated; and the storage stability and reusability of this new biocatalyst were investigated. Kinetic parameters of the immobilized laccase were investigated. Moreover, the laccase-biotitania biocatalyst particles were used for the decolorization of MG and the degradation mechanism was explored by gas chromatography-mass spectrometry (GC–MS). This study aimed to gain a deeper understanding of the capacity of laccase-biotitania particles to act as a fast biocatalyst for MG decolorization.

MATERIALS AND METHODS

Materials Laccase was purchased from Wuxi Jinkun Biology Co. Ltd. (Jiangsu, China). Titanium (IV) bis (ammonium lactato) dihydroxide (Ti-BALDH, 50 wt% aqueous solution) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and used as the titanium precursor. For the activity determination, 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) was purchased from Sigma–Aldrich. Protamine sulfate from salmon was obtained from Beijing Solarbio (Beijing, China). MG was obtained from Sinopharm Group Chemical Reagent Co. Ltd. (Beijing, China). 1-Hydroxybenzotriazole (HOBT), a mediator for enzymatic degradation of dyes, was purchased from Sigma–Aldrich. All other chemicals were analytical grade and used without further purification.

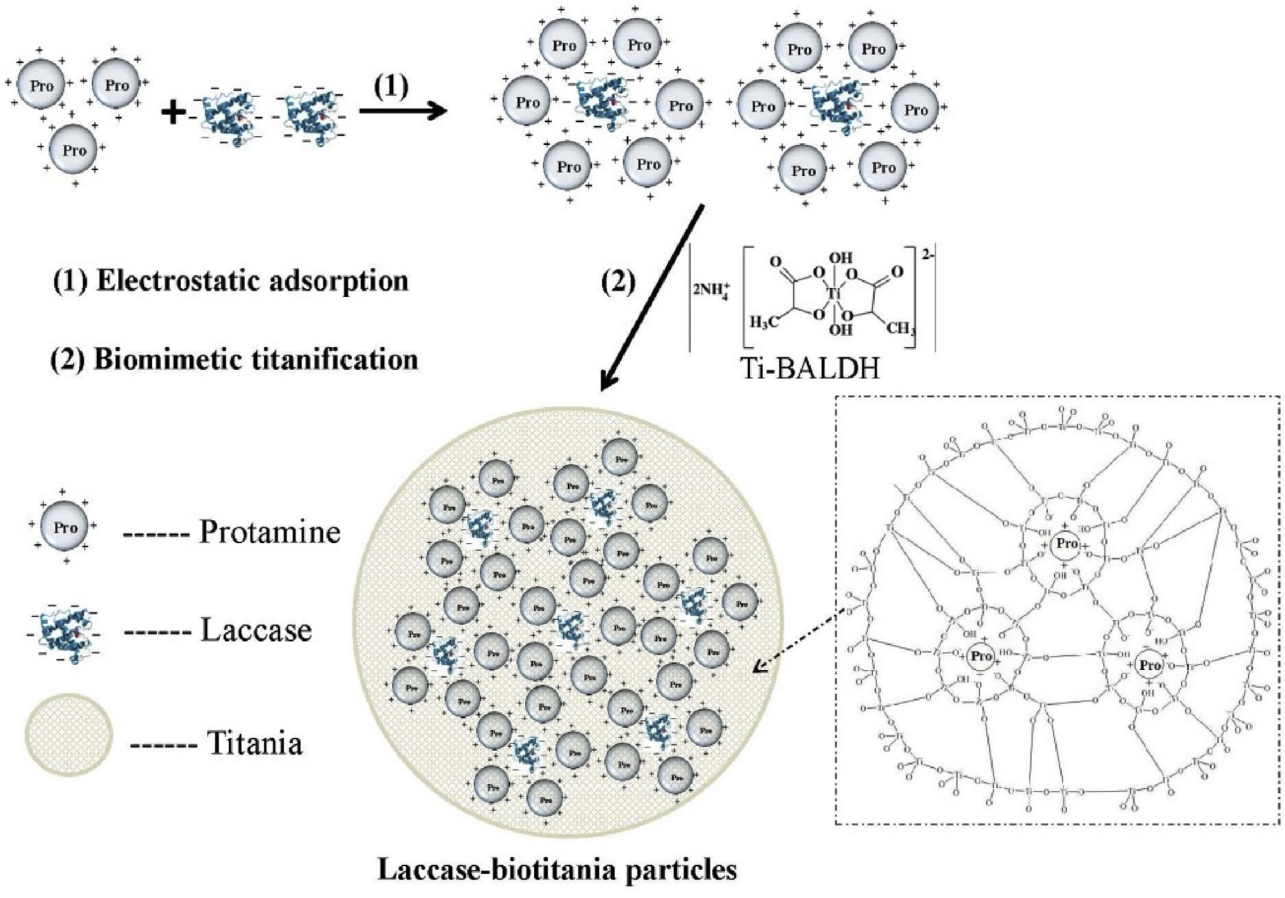

FIG. 1. Outline of the approach used in this work to produce laccase-biotitania biocatalysts via bioinspired enzyme entrapment.