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Recent developments of a co-immobilized laccase–mediator system: a review

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The laccase–mediator is a promising biocatalyst with many possible applications, including bioremediation, chemical synthesis, biobleaching of paper pulp, biosensing, textile finishing and wine stabilization. The immobilization of laccase and the mediator offers several improvements for laccase–mediator system applications because the storage and operational stabilities are frequently enhanced. Moreover, the reusability of the immobilized laccase and mediator represents a great advantage compared with the free laccase and mediator. In this work, we review the methods of co-immobilization of the laccase–mediator system for the first time systematically and comprehensively. In addition, we discuss the different methodologies of laccase and mediator immobilization that have been reported.

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1. Laccase

Laccase (*p*-diphenol: oxygen oxidoreductase, EC 1.10.3.2) is a copper-containing oxidase, and was first discovered in the Japanese lacquer tree in 1883.¹ The typical laccase molecular structure (6.5 nm × 5.5 nm × 4.5 nm) generally contains about 500–550 amino acids with molecular weights of about 50–130 kDa (Fig. 1a). Laccase can catalyze the oxidation of a wide variety of substrates, including mono-, di- and polyphenols, aminophenols, methoxyphenols, aromatic amines and ascorbate, with the concomitant four-electron reduction of oxygen to water. These enzymes couple the four single-electron oxidations of the reducing substrate to the four-electron reductive cleavage of the dioxygen bond with four copper atoms. These copper atoms are classified into three groups depending on the characteristics obtained by UV/visible and electron paramagnetic resonance (EPR) spectroscopy. Among them, the T1 copper active site is ubiquitous in all kinds of laccase, which is an important factor to determine the redox potential (0.4–0.8 V) of laccase, and is also the substrate binding site; T2 copper is a mononuclear site with normal EPR parameters, which indicates that there is a tetragonal crystal environment, and only in the UV-vis region does it show weak and unobvious absorption; T3 copper is a coupled ion pair with diamagnetism and a wide absorption band at 330 nm. The two atoms form a binuclear site. The two copper atoms on the site pair antiferromagnetically through the bridging ligand hydroxyl bridge. According to the literature,^{2–4} the coordination structures of the four copper ions are as

follows: T1 copper coordinates with two S of Cys and Phe, and two N of His, forming deformed tetrahedron; T2 copper is a trinuclear cluster, which coordinates with N and O in two His, respectively. The other two Cu atoms of T3 copper coordinate with N in three His and O in Tyr to form oxygen bridge, forming binuclear copper region (Fig. 1b).

Laccase is a single electron oxidoreductase, which catalyzes the oxidation of different types of substrates through a single electron reaction to produce free radicals, which is mainly manifested in the formation of free radicals and the synergistic effect of four copper ions in laccase molecule. Laccase could absorb redox substrate through single electron mediated by T1 copper to form free radical cation, which can further carry out laccase catalyzed oxidation. Because the oxidation of single electron substrate is paired with the four electron reduction of oxygen, it could be understood that laccase acts like a battery, storing electrons from each oxidation reaction to reduce oxygen molecules. Therefore, four substrate molecules need to be oxidized to complete the process of oxygen molecules reducing to water; each electron from the four single electron oxidation at the T1 copper site is transferred to the trinuclear copper atom center with the bound O₂ molecule, and the oxygen molecule is activated and reduced to water in the T2/T3 trinuclear domain. Typical laccase reaction mechanism is shown in Fig. 2.

The key characteristic of laccase as an oxidoreductase is that the redox potential of its redox center, *i.e.* T1, T2, T3 copper site, meets the requirements of electrochemical reaction. The research shows that T1 copper site is the initial center for most laccases to receive electrons from the reducing substrate, and the catalytic efficiency of the substrate molecule depends on the redox potential of T1 copper. Christenson⁶ reported the redox potential values of a large number of different laccase T1 copper sites determined by redox medium potentiometric titration, and pointed out that laccase can be divided into three

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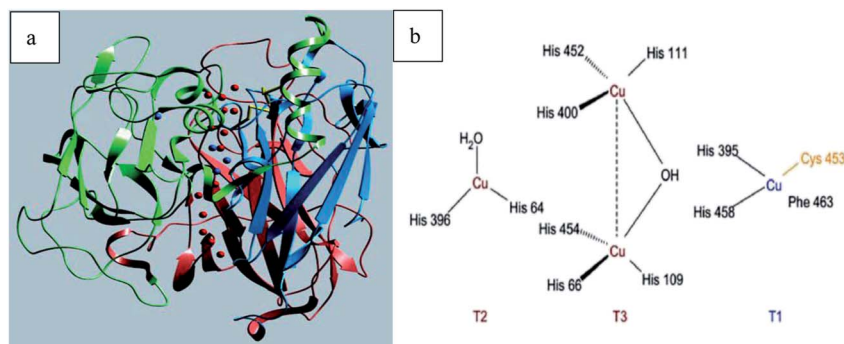



Fig. 1 The structure (a) and active center (b) of laccase.

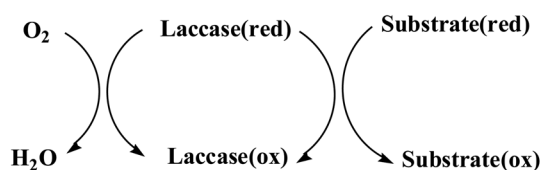


Fig. 2 Catalytic mechanism of laccase.⁵

categories according to the potential of T1 site: low, medium and high potential laccase. The low potential group includes laccase from trees, such as laccase with about 420 mV T1 copper site potential. The intermediate group included laccase from basidiomycetes, such as thermophilic mycelium, basidiomycete C30, *Rhizoctonia solani* and *Coprinopsis cinerea*. The T1 potential of these enzymes ranged from 470 mV to 710 mV. The potential of high potential laccase (such as *Trametes* (Coriolus) laccase, *T. versicolor* laccase and *T. villosa* laccase) at T1 site is about 780 mV. Based on this report, the author proposes that the limiting step of reducing substrate in the enzymatic reaction cycle is the single electron transfer of substrate to laccase T1 copper. Yaropolov⁷ studied the electrochemical behavior of two laccase from different sources on graphite electrode and carbon electrode, and obtained similar conclusion: when the electrode was modified with fungal laccase, the oxygen reduction potential was about 0.35 V higher than that of laccase from lacquer tree modified electrode, and speculated that the main reason might be due to the difference of T1 copper oxidation–reduction potential between the two laccases. Xu⁸ studied the catalytic oxidation performance of laccase for three N–OH compounds: 1-hydroxybenzotriazole, purpuric acid and *N*-hydroxyacetanilide, and concluded that the higher the redox potential of laccase, the faster the oxidation rate of substrate molecule, and the rate limiting step of laccase catalytic oxidation depends on the rate of electron transfer from substrate to T1 copper site in laccase.

2. Laccase–mediator system

Laccase could catalyze the oxidation of a variety of phenolic substrates, and has potential application value in lignocellulose degradation, environmental pollutant degradation and other

fields. It is considered to be a green environmental protection, economic and safe enzyme catalyst.^{9,10} However, there are still some problems to be solved when laccase is directly used in industrial production. Firstly, a large number of non-phenolic substrates could not directly bind to laccase; secondly, the redox potential of laccase was low (420–780 mV),⁶ and the low redox potential could not oxidize high potential phenolic compounds and other complex non-phenolic compounds. Studies have shown that the addition of some small molecular mediators can mediate the oxidation between laccase and substrate, and further expand the scope of laccase. The combination of laccase and small molecular mediators is called laccase mediator system (LMS).¹¹ In 1990, Bourbonnais¹² found that in the presence of remazol blue and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), laccase could oxidize non-phenolic lignin model compounds, and model compounds isoamyl alcohol and 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol were oxidized by laccase mediator obtaining α -carbonyl derivatives. 1-(3,4-Dimethoxyphenyl)-2-phenoxyethane-1,2-diol was cleaved in the presence of laccase and ABTS to produce veratrum aldehyde and benzaldehyde. Based on these observations, Bourbonnais concluded that laccase can oxidize non phenol lignin, but depends on the existence of mediator, so laccase–mediator system is proposed for the first time. Later, other researchers found that laccase could oxidize the non-phenolic structure unit of lignin under the synergetic action of the mediator,¹³ and could be applied to pulp bleaching.¹⁴

LMS have led to a dramatic increase in the range of laccase oxidizable compounds. Mediator oxidation by laccase produces a high redox potential intermediate able to oxidize non-phenolic substrates. This intermediate compound is then reduced to restore its initial form and close the redox cycle. The mechanism of LMS acting on substrate is shown in Fig. 3. At

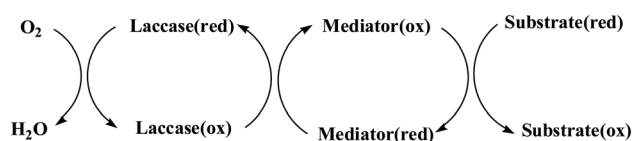


Fig. 3 Typical catalytic mechanism of LMS.



Table 1 Synthesis mediators of laccase

| Synthesis mediators | Structural formula |
|--|--------------------|
| 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) | |
| 1-Hydroxybenzotriazole (HBT) | |
| 2,2,6,6-Tetramethylpiperidyl-1-oxyl (TEMPO) | |
| N-Hydroxyacetanilide (NHA) | |
| Thiodiphenylamine (PT) | |

present, in addition to ABTS, 1-hydroxybenzotriazole (HBT), 2,2,6,6-tetramethylpiperidine oxide (TEMPO), *n*-hydroxy-*n*-acetylaniline (NHA) and phenothiazine (PT) are common synthetic mediators. The molecular structure formula is shown in Table 1.

Compared with the treatment of phenol and non phenol compounds by laccase alone, the addition of these mediators could significantly improve the catalytic oxidation efficiency of laccase to the target substrate. However, most synthetic mediators are toxic, unstable or expensive, and the large amount of use will inevitably cause secondary pollution of the environment. In addition, laccase oxidation mediator could produce a strong oxidation intermediate: the "co-mediator". In addition to acting as a diffusion electron carrier, the co-mediator also interacts with laccase, resulting in laccase passivation, which greatly reduces the biocatalytic activity of laccase.¹⁵ In order to prevent the synthetic mediator from attacking the surface activity sensitive amino acid group of laccase, it is usually used to reduce the concentration of mediator and reaction temperature, which could effectively reduce the effect of mediator on laccase activity.¹⁶ In addition, it is reported that the stability of laccase in LMS could be effectively improved by using surfactant trixon.¹⁷

In view of the potential harm of synthetic mediator to laccase and environment, researchers began to look for safer and environment-friendly natural compounds as laccase mediators. Luo¹⁸ isolated about 18 kinds of small molecular phenolic compounds from the black liquor of *Eucalyptus urophylla* kraft pulping. Most of them can be used as natural mediators of laccase and can be used in dye decolorization, oxidation of polycyclic aromatic compounds and degradation of lignin and non lignocellulosic fibers. These phenolic mediators can be rapidly oxidized by laccase because of the existence of power donor in the substituent group of benzene ring, which can

reduce the electrochemical potential energy; moreover, it can stabilize the phenoxy group of the mediator and prolong its existence time so that it can be recycled. According to reports,¹⁹ not only phenoxy compounds which could exist for a long time can act as mediators, but also their by-products. The phenoxy group of erucic acid is protected by two methoxy groups at the *ortho* position and can be specifically recognized during oxidation β - β' . The oxidized erucic acid could be rapidly converted into dehydroabietic acid endolipid, and its phenoxy group is still stable, so this phenolic dimer product could act as laccase mediator. At present, researchers have found that a variety of small molecule natural compounds can be used as laccase mediators, such as acetosyringone (As), syringaldehyde (Sa), vanillin (Va), etc. the molecular structure formula is shown in Table 2.

Compared with other synthetic mediators, natural mediators such as acetosyringone (As) and syringaldehyde (Sa) are more conducive to improve the stability of laccase and the ability to treat wastewater. The reason is that the free radical activity of these mediators after oxidation is low, which will greatly reduce the attack probability of laccase surface activity sensitive groups, so that laccase can exist stably for a long time. Camarero²⁰ pointed out that the chemical structure of As and Sa contained two methoxyl substituents on the *ortho* group of

Table 2 Natural mediators of laccase

| Natural mediators | Structural formula |
|-------------------------|--------------------|
| Acetosyringone (As) | |
| Syringaldehyde (Sa) | |
| Vanillin (Va) | |
| Acetovanillone (VO) | |
| Sinapic acid (Sad) | |
| p-Coumaric acid (p-PCA) | |



phenol, which could be rapidly oxidized by laccase. The mediator with two methoxyl substituents produced more stable phenoxy (PHO[•]) than the mediator with a single substituent. Laccase and natural phenolic mediators As and Sa were used to decolorize azo and heterocyclic dyes, and it was found that the decolorization effect of natural phenolic mediators was similar to that of synthetic mediators; when using As and Sa as mediators to investigate the decolorization performance of laccase for reactive black and sky blue, it was found that the decolorization effect of As and Sa as mediators for reactive black and sky blue was higher than that of synthetic mediator HBT.²¹ Yang²² found that acetylacetone (AA) can be used as a natural mediator to form laccase AA system, which could induce the graft polymerization of chitosan. The decolorization of malachite green by laccase with AA and HBT was compared. It was found that the degradation effect of malachite green by laccase AA was significantly better than that by laccase HBT. This may be because AA, a natural mediator, can prolong the survival rate and improve the stability of laccase. Fillat²³ found a similar phenomenon when using LMS to degrade flax pulp. Compared with using synthesis mediator HBT, natural mediator system such as Sa, As and *p*-PCA could improve the stability of laccase in the process of degrading flax pulp. Mai²⁴ also found that the natural mediator could be used as the oxidation substrate of laccase, and the natural mediator could effectively improve the stability of laccase by binding with the enzyme active center or the appropriate site in the enzyme protein chain.

3. Immobilization of laccase and mediator

3.1 Immobilization of laccase

Although laccases are excellent biocatalysts for biotechnological and environmental applications because of their high activity, selectivity and specificity, which permit them to perform complex chemical processes under experimental and natural conditions, there are some problems when free laccase is directly used in industrial production. Free laccase has strong water solubility and is not easy to be separated from the substrate products, resulting in the laccase can not be recycled after reaction, and laccase is very sensitive to the environment, easy to denaturation and inactivation. In addition, laccase is expensive, which directly leads to higher production costs,^{25,26} these problems seriously limit the practical application of laccase. Immobilization serves as an ideal technique for improving the properties and reusability of enzymes as they provide increased operational stability, thermal stability, reusability and tolerance to extreme conditions and chemical reagents. More importantly, the heterogeneity of the immobilized enzyme systems allows an easy recovery of both enzymes and products, multiple re-use of enzymes, continuous operation of enzymatic processes, rapid termination of reactions, and greater variety of bioreactor designs.

Conceptually, there are two basic methods for enzyme immobilization, as the enzyme-support link may take place by physical or chemical interactions. These different types of links

involve several types of immobilization methodologies. Physical coupling methods include the adsorption or embedding of the enzyme, whereas chemical coupling can occur through covalent binding or cross-linking method. Each of these methods has its advantages and limitations.

The adsorption of laccase onto a support is based on hydrogen bond, water dispersing bond or π -electron affinity of attraction.²⁷ This method has the advantages of environmental protection, mild conditions, simple operation, and little effect on the conformation of laccase. However, there is almost no chemical bond between the carrier and the laccase protein molecules, resulting in weak binding force and low adsorption capacity of the carrier, besides, immobilized laccase is easily affected by the changes of environmental temperature, pH and ionic strength. As a result, the adsorbed laccase molecules are easy to fall off from the surface of the carrier, resulting in the decrease of catalytic activity. Cristóvão²⁸ immobilized commercial laccase on green coconut fiber by physical adsorption method. The effects of immobilization conditions (enzyme concentration, immobilization time and pH value) on the performance of biocatalyst were investigated. It was found that the thermal stability and operational stability of laccase were improved compared with free laccase, however, the activity of immobilized laccase decreased to about 50% after reused twice. Embedding method is a kind of immobilization method that embeds laccase molecules in some material with grid or pore to form immobilized laccase. When the laccase is embedded in the carrier material, the conformation of the laccase will not be changed, so a high recovery of laccase activity can be maintained.^{29,30} However, the immobilized laccase molecules are easy to fall off from the carrier, resulting in the loss of the laccase, and there is a large mass transfer resistance between the immobilized laccase and the substrate. Zhang³¹ studied the immobilization of laccase in biomimetic titania. It was found that encapsulation of laccase in water, Tris-HCl buffer, McIlvaine buffer, and phosphate buffer had an efficiency of 80%, 97.6%, 78.5%, and 85.1%, respectively, and a recovery rate of 19.2%, 41.6%, 15.7%, and 25.5%, respectively. The reason for the differences between encapsulated efficiency and recovery of laccase activity was that the embedding method led to mass transfer limitations.

Covalent binding has been the most widely used method for laccase immobilization during the last decade. This method is based on the formation of a covalent bond between the chemical group on support or carrier and the nucleophilic group on the enzyme. Amino, imino, hydroxyl, thiol, guanidyl and imidazole groups present on the carriers are covalently linked to an enzyme. The functional group on the enzyme-like phenol ring of tyrosine, α -carboxyl group at C-terminus of enzyme and α -amino group at N-terminal of enzyme, indole ring of tryptophan is involved in the covalent binding with the support. A wide variety of supports like cellulose, agarose, collagen, inorganic carriers like glass, silica, metallic nanoparticles of gold, silver, cross-linkers like glutaraldehyde are widely used. Kashefi³² believed that covalent immobilization increases the enzyme stability and prevention of enzyme leakage to the reaction mixture and they immobilized laccase on amino-aldehyde



Jahangiri⁴² froze the mixture of poly(ethylene glycol) methacrylate, triethylene glycol diacrylate and ABTS at -20°C , and then irradiate the mixture with 12 kGy radiation dose to

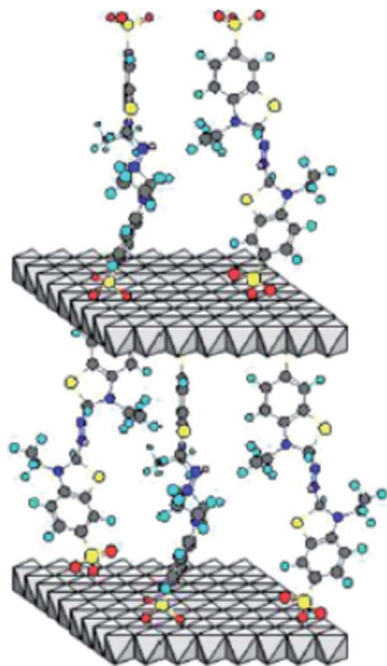


Fig. 4 Layered double hydroxide of Zn–Cr–ABTS.⁴¹

make the polymerization react to achieve the effect of cross-linking ABTS. The immobilized ABTS cold gel is used to catalyze the degradation of bisphenol A. Although the immobilization of the mediator was achieved, the immobilization efficiency of the medium was low, because the excessive radiation dose in the preparation process resulted in polymer gel depolymerization and unable to efficiently immobilize the mediator molecule. In addition, the initiation of polymerization reaction needs to be carried out under the condition of high dose radiation, which needs 10 MeV linear accelerator to accelerate the electron beam. The whole process consumes huge energy, which is contrary to the requirements of green and efficient chemical process and is difficult to be applied in practice. Sun⁴³ graft acrylamide onto the surface of chitosan through graft polymerization. Then laccase catalyze the polymerization of acrylamide and acetylacetone (AA), and laccase is embedded in modified chitosan gel by sol-gel method to obtain a co-immobilized laccase mediator biocatalyst as shown in

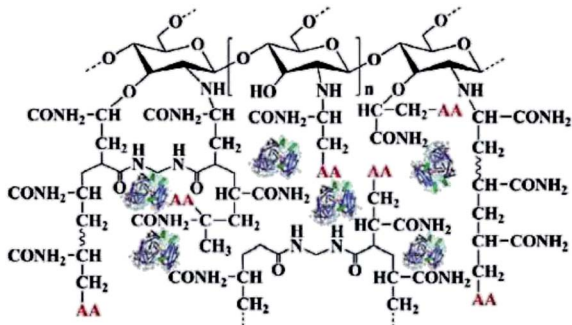


Fig. 5 Co-immobilized laccase–acetylacetone system.⁴³

Fig. 5. When the co immobilized laccase mediator catalyst was used for the biodegradation of malachite green, the degradation rate of malachite green reached 95%. However, the way of immobilizing the mediator by the polymerization of the mediator and the polymerizable monomer may lead to the change of the chemical properties of the mediator and the loss of the original redox properties of the mediator. In this process, chitosan is grafted by the graft polymerization, which may lead to low grafting efficiency and poor grafting effect due to the steric hindrance effect, in addition, the final product prepared in this report has no fixed shape and needs to be ground and crushed during use, which is inconvenient to the separation process after reaction.

Patra⁴⁴ prepared MIL-100(Fe) by microwave-assisted hydrothermal synthesis, and ABTS-MIL-100(Fe) was prepared by immersing MIL-100(Fe) in ABTS solution. The modified electrode based on ABTS-MIL-100(Fe) showed good performance in charge transfer kinetics and ionic conductivity, and had stable and repeatable electrochemical response. The results showed that MIL-100(Fe) could provide a suitable and stable microenvironment for the ABTS, and the immobilization of ABTS was realized. Then laccase was immobilized on the surface of ABTS-MIL-100(Fe) by physical adsorption method to achieve the co-immobilization of laccase–ABTS as Fig. 6. However, the immobilized ABTS prepared by impregnation method is very unstable and easy to fall off during continuous operation. The immobilization of laccase also adopts adsorption method, which causes laccase to react directly with the mediator molecules adsorbed on the surface, consumes the redox performance of the mediator, and also has a great loss of laccase activity, which will directly lead to the decrease of reusability of co-immobilized laccase–ABTS catalyst. Liu⁴⁵ also successfully packed ABTS in

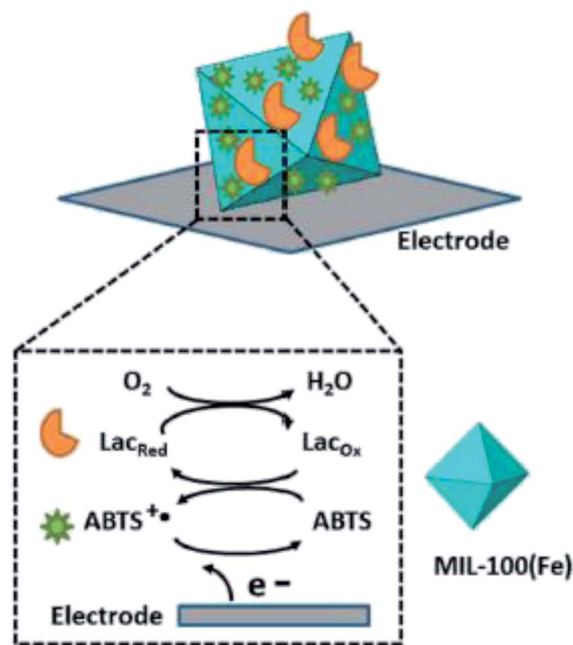


Fig. 6 MIL-100(Fe) immobilized laccase–ABTS by impregnated adsorption.⁴⁴



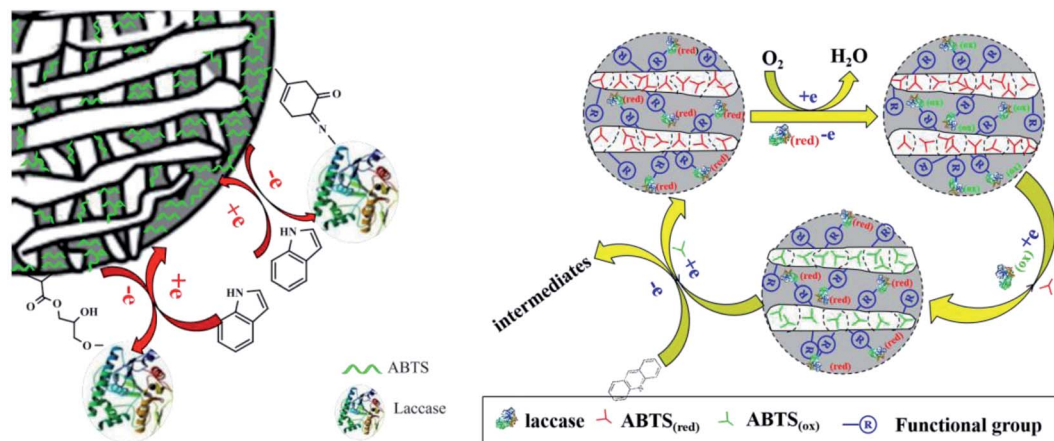


Fig. 7 Degradation of indole and acridine by co-immobilized laccase-ABTS.^{47,48}

the MIL-100(Fe) by a pot hydrothermal synthesis method obtaining ABTS-MIL-100(Fe). The ABTS-MIL-100(Fe) was used as mediator to study the removal energy of indigo Rouge dye. The decolorization rate of indigo rouge is 94% which is not different from that of free ABTS under the same conditions. While the hexagonal window size of MIL-100(Fe) is about 8.6 Å. The molecular size of ABTS is 6.4 × 6.4 × 7.4 Å, therefore, the encapsulation of ABTS molecules is not firm and easy to fall off.

Gao⁴⁶ prepared amino functionalized magnetic Fe₃O₄ nanoparticles, and immobilized laccase and mediator 4-amino-TEMPO on the surface of amino functionalized magnetic Fe₃O₄ nanoparticles through glutaraldehyde crosslinking. The co-immobilized laccase mediator catalyst was used to decolorize acid fuchsin. The decolorization rate of acid fuchsin was up to 77.41%. The results showed that the redox properties of laccase and mediator did not change after immobilization. However, glutaraldehyde as a cross-linking agent will cause partial denaturation of the enzyme molecule, because glutaraldehyde reacts strongly with the amino group of the enzyme, which may cause covalent bond connection within and between the enzyme molecular structure, resulting in the structural rigidity change of the laccase. Our group recently reported a biocatalyst that was prepared through the simultaneous immobilization of laccase and a mediator ABTS into a functionalized calcium alginate and cellulose beads (Fig. 7). This co-immobilized laccase-ABTS was then used for indole and acridine degradation.^{47,48}

4. Conclusion and future outlook

The immobilization of laccase can be performed *via* many different methods using a large number of supports. However, the methods of immobilization of mediator are not always satisfactory, let alone co-immobilized laccase-mediator system, although improvements are frequently obtained concerning the stability of the laccase-mediator to temperature, pH, organic solvents, storage and operation.

Similar to the free laccase and mediator, co-immobilized laccase-mediator can be applied in a huge number of

industrial processes, especially in environmental applications and in electrobiochemistry processes. The employment of co-immobilized laccase-mediator for the design of biological fuel cells and biosensors opens up new possibilities, from industrial to healthcare applications. The search for inexpensive supports and the recovery of activity during the immobilization process should be improved to increase the potential application of laccase-mediator co-immobilized systems.

Conflicts of interest

There are no conflicts to declare.

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