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Review

Laccase immobilization onto natural polysaccharides for biosensing and biodegradation

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ABSTRACT

Over the last few years, the focus of researchers have been set on enzyme engineering and enzyme immobilization technology using natural polysaccharides as promising and green supporting materials to address the challenges of free enzymes for various applications. Polysaccharides have been extensively implemented as enzyme carriers because they can be easily modified chemically according to the nature of immobilization. This process improves the stability and lifetime of laccase in catalytic reactions. Additionally, the selectivity of the enzymes can be preserved for particular application after immobilization onto polysaccharides. This review paper reveals the significance and potential of natural polysaccharides (including cellulose, chitosan, and alginate) and their composites as support materials for the laccases immobilization to expand the modified biocatalysts for industrial applications. Moreover, the roles of immobilized laccases are discussed from a fundamental point of view to elucidate their catalytic mechanisms as biocatalysts in the detection and degradation of environmental contaminants.

1. Introduction

Biocatalysts have recently received particular attention because of their exclusive properties in a large variety of industries (Chapman, Ismail, & Dinu, 2018; Rueda et al., 2016; Shieh et al., 2015). They are very attractive for scientists owing to minimized chemical use and the generation of nontoxic byproducts (Busto, Gotor-Fernández, & Gotor, 2010). As biocatalysts, enzymes are sustainable alternatives for conventional catalysts because they carry out the reactions with high selectivity under mild conditions (temperature, pH, pressure) (2012, Sigg et al., 2011). Laccases, copper-containing oxidoreductases (Cañas & Camarero, 2010; Giardina et al., 2010), are versatile enzymes which have been increasingly utilized in numerous industries including food, pharmaceutical, textile and paper industries as well as in biodegradation of environmental pollutants (Bilal, Iqbal, & Barceló, 2019; Bilal, Rasheed, Nabeel, Iqbal, & Zhao, 2019; Couto & Herrera, 2006; Su, Fu, Wang, Silva, & Cavaco-Paulo, 2018) and organic syntheses (Ghorashi, Shokri, Moradi, Abdelrasoul, & Rostami, 2020; Moradi, Shokri, Ghorashi, Navaee, & Rostami, 2020; Rostami, Mohammadi, Shokri, & Saadati, 2018; Shokri, Azimi, Moradi, & Rostami, 2020). However, the broad usage of laccases in industry is usually hampered by their high production costs, low stability, extreme sensitivity to pH and temperature and inconvenience in their separation, recycling, and reusing (Fathali, Rezaei, Faramarzi, & Habibi-Rezaei, 2019; Li, Xia, Li et al., 2018; Li, Xia, Niu, Ping, & Xiao, 2018). To circumvent these problems, researchers have tracked the immobilization of laccases on water-insoluble solid supports, facilitating the product purification and reusing of enzymes and, as a result, improveing the process economy (Fernandez-Fernandez, Sanromán, & Moldes, 2013; Rahmani et al., 2015; Zdarta, Meyer, Jesionowski, & Pinelo, 2019). The immobilization is also capable of increasing thermal, pH and storage stabilities of enzymes (Zdarta, Meyer, Jesionowski, & Pinelo, 2018).

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Therefore, the development of suitable support materials and methods for laccase immobilization to increase its stability and recoverability is of great importance (Bilal & Iqbal, 2019; Bilal, Iqbal et al., 2019; Bilal, Rasheed et al., 2019; Deska & Kończak, 2019). To date, numerous methods for laccase immobilization have been reported including physical (adsorption, entrapment and encapsulation) and chemical (covalent bonding and cross-linking) procedures (Bilal, Iqbal et al., 2019; Fernandez-Fernandez et al., 2013). In physical procedures, there is no requirement for the functionalization of the support; and the attachment of enzyme to carrier can be achieved via non-covalent interactions including hydrogen bonds, ligand-metal complexation, hydrophobic and electrostatic interactions (Ba, Arsenault, Hassani, Jones, & Cabana, 2013). By contrast, chemical procedures include the enzyme attachment to the functionalized support by covalent bonds, leading to the high operational stability of enzyme (Songulashvili, Jimenéz-Tobón, Jaspers, & Penninckx, 2012). In addition, there are also numerous materials that can be employed as support for laccase such as mesoporous materials (Shao et al., 2019), carbon nanotubes (Tavares et al., 2015), inorganic oxide nanoparticles (Ji, Nguyen, Hou, Hai, & Chen, 2017), and so on (Deska & Kończak, 2019; Rouhani, Rostami, & Salimi, 2016). Among the applied supports, polysaccharides are of great importance owing to high natural abundance, low cost, thermal and mechanical resistance, non-toxicity, biocompatibility, biodegradability, and renewability (Yang, Han, Zheng, Dong, & Liu, 2015). Regarding the structural properties, it is noteworthy that polysaccharides are carbohydrate macromolecules with natural (animal, plant, algal, microbial) origins composed of glycosidic linkages of monosaccharide repeating units in linear or branched fashion (Seidi, Salimi, Shamsabadi, & Shabanian, 2018; Yadav & Karthikeyan, 2019). In addition, they possess numerous reactive functional groups in their chemical structure (e.g. hydroxyl, amino, and carboxylic acid groups), resulting in high binding affinity of these compounds for enzymes (Bilal & Iqbal, 2019). Due to the abundant number of OH groups and polyionic nature of some types of polysaccharides (such as chitosan and alginate), physical immobilization of laccase onto the polysaccharide-based supports generally requires no pre-treatment. On the other side, despite the presence of various functional groups in the structure of polysaccharides, sometimes further pre-modifications are required to insert the desired functional groups (such as —SH, —CH═O,...) onto the backbone of polysaccharide. These functional groups enable polysaccharide supports for covalent conjugation to laccase. Covalent conjugation is performed generally by formation of Schiff base, amide, and disulfide linkages between laccase and polysaccharide-based support.

Taking into account the mentioned background, the main purposes of this review are to: (a) evaluate challenges of the free laccases for industrial applications; (b) help in better understanding of laccases immobilization; (c) elucidate the advantages of utilizing the natural polysaccharides including cellulose, alginate, chitosan (CS) and their composites as green supports for laccases immobilization; (d) classify applications of immobilized laccases onto the polysaccharides; (e) clarify catalytic mechanism of immobilized laccases as biocatalysts in various industrial applications.

2. Characteristics of laccases

Laccases belong to the group of multi-copper oxidases which were discovered by Yoshida in 1883 (Yoshida, 1883). They are usually produced by plants, fungi, several bacteria strains and insects. Laccases encompass four copper atoms categorized by electronic paramagnetic resonance (EPR) and UV/visible spectroscopy into three redox groups: Type 1 (T_1), Type 2 (T_2) and Type 3 (T_3) (Burton, 2003; Catherine, Penninckx, & Frédéric, 2016; Kunamneni, Plou, Ballesteros, & Alcalde, 2008; Mogharabi & Faramarzi, 2014). Type 1 Cu, which makes the blue color of laccase, displays strong EPR signals and an intense peak at 600 nm. Type 2 Cu has no absorption in the visible spectra, however it is EPR active. Type 3 coppers include a pair of Cu atoms that are characterized by a weak absorbance at 330 nm with no EPR signal.

3. Laccase-catalyzed oxidations

Laccases oxidize numerous substrates including phenolic compounds, aliphatic and aromatic amines, hydroxylamines and benzenethiols under mild conditions using O_2 and generating H_2O as the only byproduct (Ashrafi, Nasseri, Alimohammadi, Mahvi, & Faramarzi, 2016; Senthivelan, Kanagaraj, & Panda, 2016; Taghizadeh et al., 2020). Owing to their capability for oxidizing the phenolic compounds, laccases have been broadly utilized in wastewater remediation, paper and pulp processing, food preparation and bioremediation (Couto & Herrera, 2006).

Laccase-assisted oxidation comprises three major steps (Mate & Alcalde, 2015): (i) T_1 Cu is reduced by the electron released from an oxidized substrate; (ii) the electrons are transferred from T_1 Cu to T_2/T_3 site; (iii) lastly, electrons are employed to reduce O_2 to H_2O at T_2/T_3 site.

3.1. Laccase-catalyzed oxidation of phenolic compounds

Substrates directly oxidized by laccase are compounds with lower redox potentials than laccase (range, 400−800 mV). Therefore, phenols are considered as the main substrates of laccase-catalyzed oxidations. Oxidation of phenolic compounds using laccase is presented in Fig. 1 (Su et al., 2018). As it is shown, phenols are initially oxidized to phenoxyl radicals, which undergo oxidative coupling with formation of oligomers and polymers. Moreover, the resulting radicals can also be transformed into dead end products via the radical rearrangement. Meanwhile, reversibility of the oxidation depending on radical intermediates stability may also be occurred.

3.2. Laccase-catalyzed oxidations employing mediators

Typically, laccases alone cannot oxidize non-phenolic compounds owing to their high oxidation potentials. It is worth to mention that the laccase/mediator systems (LMS) may resolve this problem (Hilgers, Vincken, Gruppen, & Kabel, 2018; Morozova, Shumakovich, Shleev, & Yaropolov, 2007). Notably, laccase initially reacts with the mediator and then the obtained intermediate oxidizes the corresponding substrate. The best-known mediators are as follows: *N*-hydroxybenzotriazole (HBT) (Sun, Huang, & Li, 2017), violuric acid (VA) (Asif, Hou, Price, Chen, & Hai, 2020), 3-hydroxyanthranilic acid (HAA) (Feng et al., 2019), *N*-hydoxy-phthalimide (HPI) (Munk, Andersen, & Meyer, 2018), 2,2′-azinobis-(3-ethylbenzylthiozoline 6-sulphate) (ABTS) (Xue et al., 2020), and 2,2,6,6-tetramethyl-1-piperid-inyloxyl (TEMPO) (Risi, Zhao, & Castagnolo, 2019).

4. Immobilization of laccase

The usage of free laccases in industrial processes faces problems such as high production prices, inadequacy in enzyme separation and reusability as well as extreme sensitivity to environmental factors like pH, temperature, and inhibitors, resulting in poor operational stability and rapid loss of activity (Fathali et al., 2019; Li, Xia, Niu et al., 2018). These challenges are overcome by the immobilization of laccases on water-insoluble supports (Deska & Kończak, 2019; Ji et al., 2017; Rouhani et al., 2016; Shao et al., 2019; Tavares et al., 2015). Immobilization, a process through which the enzyme is attached to a solid support, converts the form of the catalyst from homogenous (free enzyme) to heterogenous (immobilized enzyme) (Zdarta, Meyer, Jesionowski, & Pinelo, 2018). The interactions between the enzyme and support in this process stabilize the peptide structure of the biocatalyst and, as a result, improve the enzyme stability towards temperature, pH, storage, operation and chemical reagents (Zdarta et al., 2018a). In addition, immobilization also facilitates the purification steps and thus generates desired products with higher yields (Zhou, Zhang, & Cai, 2020). However, the main advantage of immobilization is the

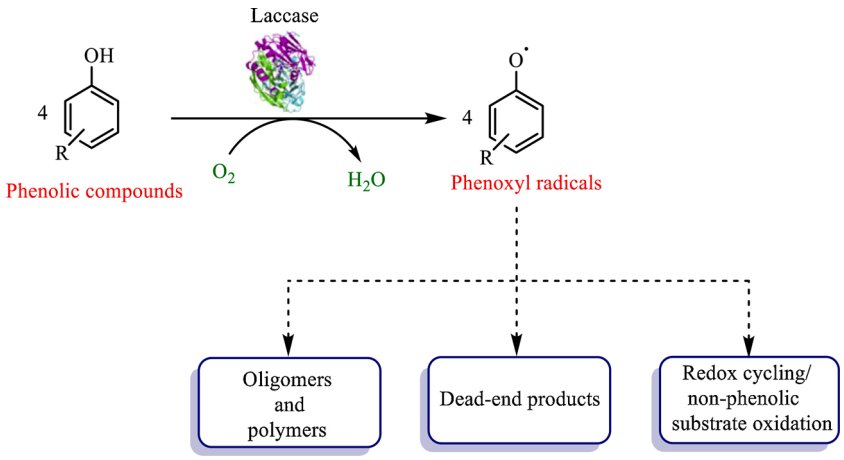

Fig. 1. Direct oxidation of phenolic compounds using laccase (Su et al., 2018).

production of an enzymatic system that can be reused in many catalytic cycles without significant loss of its unique properties, which will reduce the operational cost that is a key factor from the economical point of view (Zhou et al., 2020). The low-price, green nature, renewability, eco-friendly, functionability and diversity of polysaccharides make them as fantastic support materials for laccase with great potential of being used in industry.

Immobilization techniques for laccases are classified in two main groups, physical and chemical procedures (Fernandez-Fernandez et al., 2013) which both category have been used for immobilization of laccase on the polysaccharide supports.

The physical procedures include entrapment, adsorption and encapsulation, which keep the enzyme's catalytic activity since the enzyme conformation is not altered during the immobilization process (Fernandez-Fernandez et al., 2013). Adsorption-based immobilization is an easy and economical method in which the enzyme is attached to the exterior surface of carrier through weak interactions including hydrogen bonds, electrostatic and hydrophobic interactions (Zhou et al., 2020). In the case of polysaccharide-based supports, due to the absence of hydrophobic groups, the physical immobilizations mainly involve H-bondings and electrostatic interactions. Because of these weak interactions, this technique displays some drawbacks such as leaching of the enzyme from the carrier during its application and poor stability against pH and temperature (Zhou et al., 2020). In entrapment and encapsulation immobilizations, enzyme is occluded in a polymeric network (e.g. alginate, carrageenan, hydrogel structures, alginate-chitosan microcapsules) while the substrates pass through the network for the reaction to take place (Fernandez-Fernandez et al., 2013). Accordingly, the mechanical and operational stabilities of the enzyme are enhanced. However, the main limitation of these techniques is the existence of the high diffusion barrier which prevents passing macromolecular substrates.

By contrast, the chemical methods comprise of covalent bonding and cross-linking techniques, which rely on the formation of covalent bonds between enzyme and the carrier (Fernandez-Fernandez et al., 2013). Immobilization by covalent bonding includes the reaction of functional groups of the carrier with functional groups of the enzyme. This method can be accomplished by a single step process when the functional group of the carrier does not require any modification (Karthik et al., 2021). Otherwise, it requires pre-modification of the carrier with a proper functional group to capable it for covalent conjugation to the enzyme (Karthik et al., 2021). The covalent linkage between the enzyme and carrier significantly decreases the leaching of enzyme and increases its thermal stability and reusability (Songulashvili et al., 2012). However, the chemical association changes the conformational of the enzyme and decreases the catalytic activity (Jun et al., 2019). Of note, cross-linking immobilization combines both covalent bonding and entrapment techniques and requires cross-linking agents such as glutaraldehyde and bisisodiacetamide (Eş, Vieira, & Amaral, 2015).

Beyond the above-mentioned restrictions, another major obstacle using free laccase as the catalyst is the low redox potential (0.4–0.8 V). It cannot directly oxidize the compounds with high redox potential, leading to the limitation in the range of substrates for laccase action. It is found that the redox potential of laccase can be expanded in the presence of redox mediators such as HBT, VA, HAA, HPI, ABTS, TEMPO, and so on (Shokri et al., 2020). Although the use of laccase-mediator systems (LMS) extend the range of laccase substrates, the mentioned mediators are quite expensive, highly toxic and difficult to recover and reuse in the soluble states (Shokri et al., 2020). Therefore, the major limiting factors of free laccase and dissolved mediators for large-scale application are low stability and reusability and high cost of the enzyme and mediators accompanied by the potential toxicities of dissolved mediators (Huang, Yang, Wang, Zhang, & Liu, 2018). In this regard, the co-immobilization of laccase and mediator on water-insoluble carriers has been recognized as the most efficient strategy to overcome these limitations (Yaohua, Ping, Feng, & Keren, 2018).

The facile and effective separation of catalyst from the treatment mixture in industry is important. Magnetization of the supports using various types of iron oxides has been introduced as a safe and effective method for improving the separation process of catalyst with the induction of a magnetic field (Darwesh, Matter, & Eida, 2019; de Oliveira et al., 2020; López et al., 2019). This magnetic behavior significantly reduces processing time and simplifies the enzymatic reaction and recovering the enzymes for their continual uses, especially in industrial applications (Bilal, Rasheed, Iqbal, & Yan, 2018; Bilal, Zhao, Rasheed, & Iqbal, 2018). The iron oxide magnetic nanoparticles (e.g. Fe_3O_4 and γ-Fe_2O_3) possess the unique features of nano-materials along with plentiful hydroxyl groups on their surface that make their modification possible with different materials such as silica, metal organic frameworks (MOFs), biopolymers and mesoporous materials (Wong, Tan, Lau, Yap, & Danquah, 2019). Combination of these magnetic nanoparticles with polysaccharides can afford green and easy separable supports for laccase which will be discuss in the next sections in detail.

After presenting a general overview of immobilization techniques, natural polysaccharides employed as carrier for laccase immobilization and their applications are explained in the next sections.

5. Polysaccharides as support materials for laccase immobilization

Among various polysaccharides, cellulose, chitosan, alginate, and their derivatives are the ones frequently applied as supports for laccases.

Table 1
Polysaccharide-based supports for laccase immobilization and the corresponding applications.

Laccase source	Support	Immobilization strategy	application	Ref
Cellulose				
Aspergillus oryzae	CA/BMI·N(Tf)$_2$	Physical entrapment	Sensor for methyldopa	(Moccelini et al., 2011)
T. versicolor	GR-CMF	Physical entrapment	Sensor for CC	(Palanisamy et al., 2017)
Pleurotus florida NCIM 1243	Dialdehyde CNFs	Schiff base linkages	Dyes degradation	(Sathishkumar et al., 2014)
n.a.[a] (Sigma)	Dialdehyde BC/TiO_2	Schiff base linkages	ABTS oxidation; UV-triggered dye decolonization	(Li et al., 2017)
Myceliophthora thermophile	PDA/BC	Reaction of the amino groups of laccase with quinone units in PDA.	Dye degradation	(Zhai et al., 2019)
Trametes villosa	TEMPO-oxidized CA	Amide formation between -COOH groups of TEMPO-oxidized CA with -NH_2 groups of laccase	Degradation of combustion toxicants including resorcinol, hydroquinone and methylcatechol	(Prasetyo et al., 2015)
n.a. (Sigma-Aldrich)	CNFs/PEI/GA	Schiff base linkages	Trifluralin degradation	(Bansal et al., 2018)
T. versicolor	Cellulose beads functionalized by epoxides and quinones	Reaction of -NH_2 groups of laccase with epoxides and quinones on the cellulose sruface	Detoxification of nitrogenous organic compounds (indole & carbazole)	(Gu et al., 2020; Yaohua et al., 2018)
n.a. (Taiwan biological company in Taipei)	The carboxylated cellulose beads reinforced by CF	Electrostatic interaction	Degradation of 4-hydroxy-3,5-dichlorobiphenyl	(Li, Xia, Li et al., 2018)
Chitosan				
T. versicolor	CS/ NCHS	Physical entrapment	Sensor for kraft lignin	(Li et al., 2012)
T. versicolor IBL-04	GA-crosslinked CS microspheres	Schiff base linkages	Dyes degradation	(Asgher et al., 2017)
Trametes pubescens	GA-crosslinked CS microspheres	Schiff base linkages	Dyes degradation	(Zheng et al., 2016)
T. versicolor	CS/GA macrobeads	Schiff base linkages	Anthracene detoxification	(Apriceno et al., 2017)
T. versicolor	GA-crosslinked CS/CeO_2 microspheres	Schiff base linkages and adsorption	Dyes degradation	(Lin et al., 2015)
T. versicolor	CS/TPP/GA	Schiff base linkages	Detoxification of tetracycline	(Jeong & Choi, 2020)
Trametes pubescens	Genipin-crosslinked CS beads	Physical entrapment	Dye decolonization	(Ma et al., 2018)
T. versicolor	Epoxy-functionalized magnetic CS	Reaction of -NH_2 moieties of laccase with epoxide on the carrier sruface	Dyes decolonization	(Bayramoglu et al., 2010)
Weissella viridescens LB37	GA-crosslinked magnetic CS	Schiff base bonds	Degradation of synthetic dyes	(Nadaroglu et al., 2019)
Trametes trogii	Fe_3O_4-TCS composite	Disulfide linkages	Decolonization of textile dyes	(Ulu et al., 2020)
n.a.	CS-coated Fe_3O_4/GA	Schiff base linkages	Detoxification of chlorophenols	(Zhang et al., 2020)
T. versicolor	Magnetic CS-clay/GA	Schiff base linkages	Phenol treatment	(Aydemir & Güler, 2015)
T. versicolor	HNTs-Fe_3O_4-CS/GA	Schiff base linkages	Dye decolonization	(Kadam et al., 2018)
T. versicolor	HNTs-Fe_3O_4-CS (1%)/GA	Schiff base linkages	Detoxification of SMX	(Kadam et al., 2020)
T. versicolor	CS/PVA/GA	Schiff base linkages	Biodegradation of 2,4-DCP	(Xu et al., 2013)
T. versicolor	PA6/CS-GA-BSA-GA and PA6/CS-GA-HMD-GA	Schiff base linkages	Degradation of EDCs	(Maryšková et al., 2016)
T. versicolor	pIA grafted and Cu(II) chelated CS membranes	Electrostatic interaction	Dyes removal	(Bayramoglu et al., 2012)
T. versicolor	CS/PGMA/PEI/Cu(II)	Physical adsorption	Phenol removal	(Alver & Metin, 2017)
Alginate				
Ganoderma sp. KU-Alk4	Cu(II)-alginate beads	Physical entrapment	Dyes decolonization	(Teerapatsakul et al., 2008) (Teerapatsakul et al., 2017)
Coriolopsis gallica	Ca(II)-alginate beads	Physical entrapment	Textile dyes treatment	(Daâssi et al., 2014)
Streptomyces psammoticus	Cu(II)-alginate beads	Physical entrapment	Phenolic compounds detoxification	(Niladevi & Prema, 2008)
Lentinus polychrous	Ba(II)-alginate	Physical entrapment	Acetaminophen removal	(Ratanapongleka & Punbut, 2018)
T. versicolor	*n*Fe_2O_3/Cu(II)-alginate hybrid system	Encapsulation	Remediation of triclosan and RBBR dye	(Le et al., 2016)
Aspergillus oryzae	ABTS-LDH/alginate beads	Physical adsorption	Dye removal	(Huang et al., 2018)
T. versicolor	Ca-alginate/PGMA/ PDA/ABTS	The reaction of amino units on the enzyme with the quinone and epoxy units on the carrier	Biodegradation of acridine	(Xue et al., 2020)

Abbreviations: cellulose acetate (CA), 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide (BMI·N(Tf)$_2$), *Trametes versicolor* (T. versicolor), graphene-cellulose microfibers (GR-CMF), catechol (CC), cellulose nanofibers (CNFs), bacterial cellulose (BC), polydopamaine (PDA), polyethylene imine (PEI), cellulose fibril (CF), chitosan (CS), nitrogen-doped carbon hollow spheres (NCHS), tripolyphosphate (TPP), thiolated chitosan (TCS), halloysite nanotubes (HNTs), sulfamethoxazole (SMX), polyvinyl alcohol (PVA), 2,4-dichloro-phenol (2,4-DCP), polyamide 6 (PA6), bovine serum albumin (BSA), hexamethylenediamine (HMD), endocrine-disrupting chemicals (EDCs), polyitaconic acid (pIA), poly(glycidyl methacrylate) (PGMA), Remazol Brilliant Blue R (RBBR), layered double hydroxide (LDH).

[a] n.a. : not available.

Table 1 summaries the various techniques applied for immobilization of laccases on polysaccharides along with the applications of the obtained hybrid systems.

5.1. Cellulose

Cellulose, a linear polysaccharide produced by plants, comprises of glucose molecules connected through of β-1,4-glucosidic bonds (Salimi, Aryanasab, Banazadeh, Shabanian, & Seidi, 2016). Cellulose, as the most plentiful natural biopolymer on earth, has been efficiently used as support for laccase immobilization because it is extremely inert, economical, biodegradable and environmentally benign (Karami, Zeynizadeh, & Shokri, 2018; Li, Xia, Li et al., 2018; Movagharnegad, Najafi Moghadam, Nikoo, & Shokri, 2018). More importantly, the existence of abundant primary or secondary hydroxyl groups in cellulose structure not only make opportunities for cellulose surface modification but also facilitate adsorption or covalent attachment of enzyme to this support (Li, Xia, Li et al., 2018). The laccase/cellulose hybrid systems have mainly been utilized as biosensors and also for degradation of organic pollutants including dyes and xenobiotics.

Biosensors have appeared as analytical devices which show high rapid, accuracy, sensitivity and selectivity for the recognition of analytes (Bollella & Katz, 2020; Pinyou, Blay, Muresan, & Noguer, 2019). Among enzymes, laccases are proper candidates for biosensing applications because of good stability, the ability to carry out electron-transfer reactions and to catalyze the oxidation of phenols with aerial oxygen. Accordingly, laccase-based biosensors have been successfully implemented in the recognition of phenolic compounds (Rodríguez-Delgado et al., 2015). Within this context, Moccelini et al. entrapped laccase in the mildly calcined cellulose acetate/1-butyl-3-methylimidazolium bis (trifluoromethylsulfonyl) imide (CA/BMI·N(Tf)$_2$) support and then inserted the obtained biocatalyst into a carbon paste electrode (CPE) (Fig. 2a) (Moccelini, Franzoi, Vieira, Dupont, & Scheeren, 2011). The obtained biosensor demonstrated high recognition efficiency for the methyldopa at concentrations of ~ 35–370 μM. Meanwhile, owing to synergistic influence between the enzyme and carrier, this biosensor also displayed significant repeatability and stability for substrate determination. In regard with mechanism of reaction, the laccase firstly oxidized the methyldopa to quinone, which was then converted to methyldopa on the surface of the electrode (Fig. 2b).

In another effort, a novel biosensor was constructed via the immobilization of laccase on the modified carbon electrode with graphene-cellulose microfibers (GR-CMF) (Palanisamy et al., 2017). The prepared biosensor showed higher sensitivity (0.93 $\mu A \mu M^{-1}$ cm^{-2}) and selectivity for the detection of the catechol (CC) than other laccase subtracts (e.g. mono, di, polyphenol and aminophenol compounds). It is noticeable that CC has been known as a hazardous organic pollutant because of its high toxicity and poor biodegradability. Fig. 2c shows the plausible mechanism of CC oxidation by this biosensor. Moreover, the as-prepared biosensor also exhibited a superb electrocatalytic activity in the concentration range ~ 0.2–208 μM along with a low detection limit (85 nM) for CC. Notably, the fast response (2 s), excellent storage stability paired with good reproducibility and recovery are the main advantages of this biosensor.

Laccase-based catalysts have also been widely used for removal of organic pollutants from various wastewater resources. Dyes, as substances with various chemical structures, have been broadly utilized in textile, cosmetics, paper, plastic, printing, food, and pharmaceutical industries (Bilal, Rasheed et al., 2018; Bilal, Zhao et al., 2018; Yadav & Yadav, 2015). These compounds have been considered as environmental contaminants owing to high toxicity, stability and resistance to degradation (Jun et al., 2019). Xenobiotics as another organic pollutants are existent in, but foreign to, organisms and the environment (Koppel, Rekdal, & Balskus, 2017). These compounds consist of pesticides, petroleum compounds, pharmaceuticals, polycyclic hydrocarbons, nitrogenous compounds and etc. (Gianfreda & Rao, 2008; Maculewicz et al., 2020; Szczepańska, Kudłak, & Namieśnik, 2018). They come into the environment via numerous industrial and agriculture activities (Gianfreda & Rao, 2008). Recently, the contamination of environment by xenobiotic compounds has become a serious subject since these compounds display high toxicity (Bajaj, Chowdhury, Yucha, Kelly, & Xiao, 2018; Farag & Alagawany, 2018). Consequently, it is vital to eliminate dyes and xenobiotics from industrial wastewaters. Nowadays, laccases have been recognized as green biocatalysts for the decolorization of wastewaters (Deska & Kończak, 2019; Fernandez-Fernandez et al., 2013) and detoxification of xenobiotics (Bilal, Iqbal et al., 2019; Taheran et al., 2017).

Sathishkumar and coworkers in 2014 reported the tethering of laccase to electrospun cellulose nanofibers (CNFs) and evaluated the capability of the resulted system for dyes decolonization (Sathishkumar et al., 2014). To this end, CNFs were firstly oxidized using $NaIO_4$ to create aldehyde moieties, which were served for laccase connection through Schiff base formation between the −CHO moieties of the nanofibers and $—NH_2$ moieties of laccase. Compared to

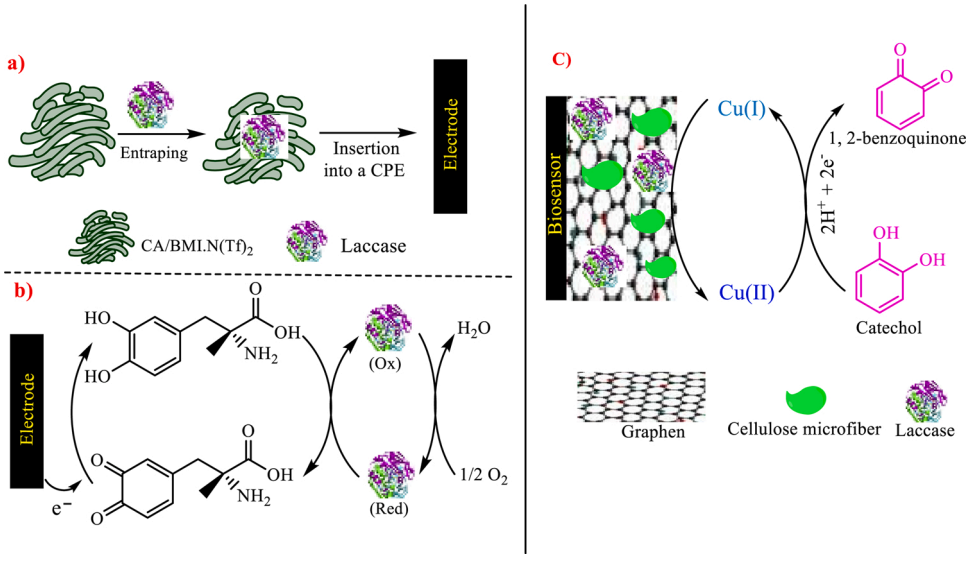

Fig. 2. a) The synthetic steps of laccase biosensor based on CA/BMI·N(Tf)$_2$; b) the methyldopa oxidation using the laccase biosensor based on CA/BMI·N(Tf)$_2$ (Moccelini et al., 2011); and c) the mechanism of CC oxidation using the laccase biosensor based on GR-CMF modified carbon electrode (Palanisamy et al., 2017).

non-immobilized enzyme, CNFs-assisted laccase displayed higher pH and thermal steadiness. Table 2 presents the outcomes of various dyes and simulated dye effluent (SDE) degradation by non-immobilized and immobilized laccase. As can be seen, the immobilization did not change the activity of enzyme. The tested dyes (except RBBR) and SDE were eliminated with low yields using non-immobilized and immobilized laccase (Table 2). However, in the existence of HBT mediator the removal of all dyes and SDE was improved significantly. A removal of 99% for SDE was acquired by 6.14 U/mL of laccase and 0.9 mM of HBT. Meanwhile, the immobilized laccase showed excellent reusability with ability to degrade 85 % of SDE at 8th run.

Similarly, the covalent immobilization of laccase onto the $NaIO_4$-oxidized bacterial cellulose (BC) in the presence of TiO_2 nanoparticles yielded a composite membrane for photo and bio-degradation of dyes (Li et al., 2017). The resulting system showed higher pH and temperature stability than free enzyme and preserved 67 % of its catalytic performance for ABTS oxidation after 10 runs. Moreover, it also displayed excellent efficiency (95 %) via a combination of the biocatalytic feature of laccase and the photocatalytic feature of TiO_2 in the decolonization of X-3B dye under UV irradiation.

Recently, polydopamaine nanoparticles/bacterial cellulose (PDA/BC) composite was prepared by polymerization of dopamine onto BC and employed to attach covalently laccase via the Michael addition or imine formation with quinone moieties (Fig. 3) (Zhai, Chen, Jin, & Hu, 2019). After immobilization, a noteworthy improvement in thermal and operative stability and catalytic activity of laccase was observed. When 0.1 U/mL of the biocatalyst was utilized, 91 % of RBBR dye was degraded within 3 h, which was considerably greater than that of non-immobilized counterpart. Besides, the reusability of the as-prepared biocatalyst for RBBR removal showed that after 5 runs, almost 65 % of RBBR removal was attained.

Partial deacetylation of CA following by carboxylation with TEMPO and activating of the carboxyl groups by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) provide a proper support for covalent immobilization of laccase through formation of amide bonds (Prasetyo, Semlitsch, Nyanhongo, Lemmouchi, & Guebitz, 2015). The resulted biocatalyst from this procedure presented high catalytic activity for oxidizing ABTS. Furthermore, it also degraded above 60 % of resorcinol, hydroquinone and methylcatechol combustion contaminants. In general, the laccase-modified CA can be a viable candidate for the toxicants treatment during combustion in numerous industries.

Bansal and his group linked laccase with 85 % yield to CNFs, by pre-activating of CNFs with epichlorohydrin and subsequent functionalization with polyethylene imine (PEI), GA and Cu(II), and then studied the catalytic activity, stability and reusability of the resulted biocatalyst in the ABTS oxidation (Bansal, Kumar, Chauhan, & Kaushik, 2018). GA conjugated the laccase to the CNF/PEI hybrid system via imine linkages (Fig. 4). The biocatalyst retained 75 % of initial catalytic activity after 45 days as well as 15 cycles of reuse led to ~ 61 % of its original activity. This biocatalyst has shown high stability which was related to strong covalent association between laccase and CNFs/PEI. As well, the biocatalyst was able to eliminate trifluralin, a toxic pesticide, with 100 % and 78.7 % yields within 24 h using guaiacol (GUA) and CC mediators, respectively.

In an alternative strategy, surface of cellulose beads was functionalized with epoxide, quinone and CC units by grafting of poly(glycidyl methacrylate/methylene bisacrylamide) on the cellulose surface and then partial reaction of the epoxide units with dopamine (Gu, Xue, & Shi, 2020). Laccase was immobilized on the beads surface by reaction with epoxides and quinone units (Fig. 5a). The developed biocatalyst displayed improved pH and temperature stability paired with the potential capability for the detoxification of nitrogenous organic compounds. Consequently, it eliminated almost 100 % of indole and 97.4 % of carbazole using ABTS (2 mg) at pH 5 and 30 °C. It is worth noticing that this biocatalyst also showed supreme reusability since around 95 % of indole and 66 % of carbazole were detoxified after 10 runs. In the extension of this work, the same cellulose beads were applied for the co-immobilization of laccase and ABTS mediator to treat indole (Yaohua et al., 2018). For this purpose, ABTS molecules were initially encapsulated into the functionalized cellulose beads, followed by the covalent tethering of laccase through reaction with inserted epoxides and quinones. The obtained biocatalyst resulted in 100 % degradation of indole (the identical result with the above-mentioned instance), whereas indole is barely degraded by free laccase. Moreover, it presented noteworthy storage stability and reusability for the indole degradation, probably related to the mediation influence of immobilized mediator. The effective recovery of both expensive laccase and hazardous ABTS provides an opportunity for utilizing laccase in the remediation of industrial wastewater. In addition, Fig. 5b indicates the proposed mechanism of indole degradation using the immobilized laccase-mediator system.

Immobilization of laccase on the carboxylated cellulose beads reinforced by cellulose fibril (CF) provided a proper system for water treatment through degradation of 4-hydroxy-3,5-dichlorobiphenyl (HO-DiCB) (Li, Xia, Li et al., 2018). Electrostatic adsorption among −COOH groups of the cellulose beads with $-NH_2$ groups of laccase was the main driving force for the enzyme immobilization. Remarkably, the obtained biocatalyst displayed enhanced thermal and operative steadiness than the free counterpart. Besides, it was also successful in degrading 85 % of HO-DiCB (20 mg/L) from effluents.

5.2. Chitosan (CS)

CS, a linear polysaccharide comprised of connected β-(1,4)-glucosamine, is acquired commercially via the deacetylation of chitin from lobster and shrimp shells (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Rinaudo, 2006). In addition, it can be also generated directly by several fungi (Seidi et al., 2018). Presence of amino and hydroxyl groups in the CS structure give it high potential for diverse types of modifications (Seidi et al., 2018). CS-based materials have been well-known as supports for enzyme immobilization due to their attractive characteristics including widespread availability, low cost, non-toxicity, mechanical stability, biodegradability, and high affinity to enzymes (Krajewska, 2004; Metin, 2013). Similar to the cellulose-based supports, covalently and physically immobilized laccases on the CS-based supports have been used for biosensing, dyes degradation, and xenobiotics detoxification.

In this regard, an amperometric biosensor with high sensitivity and storage stability was manufactured through entrapping laccase into the CS/nitrogen-doped carbon hollow spheres (NCHS) for electrochemical detection of kraft lignin by the aid of ABTS mediator (Li, Cao, Qian, Chen, & Liu, 2012). NCHS was prepared by heat treatment of poly (*o*-phenylenediamine) hollow spheres. An extensive detection range (0.37−19 mM) paired with a recognition limit of 0.12 mM for this substrate was achieved.

The catalytic functionality of *T. versicolor* IBL-04 laccase (Asgher, Noreen, & Bilal, 2017) and *Trametes pubescens* laccase (Zheng et al., 2016) were improved upon immobilization on the GA-crosslinked CS microspheres. The insolubilized laccase indicated the significant

Table 2
The degradation of various dyes and SDE using non-immobilized and immobilized laccase (Sathishkumar et al., 2014).

Type of dye	Degradation (%)	
	Non-immobilized laccase	Immobilized laccase
Remazol Black 5	49 ± 2	47 ± 4
Remazol Brilliant Blue R (RBBR)	94 ± 3	90 ± 3
Remazol Brilliant Violet 5R	13 ± 1	12 ± 2
Reactive Orange 16	20 ± 4	20 ± 4
Reactive Red 120	21 ± 3	20 ± 3
Simulated dye effluent	39 ± 4	37 ± 3

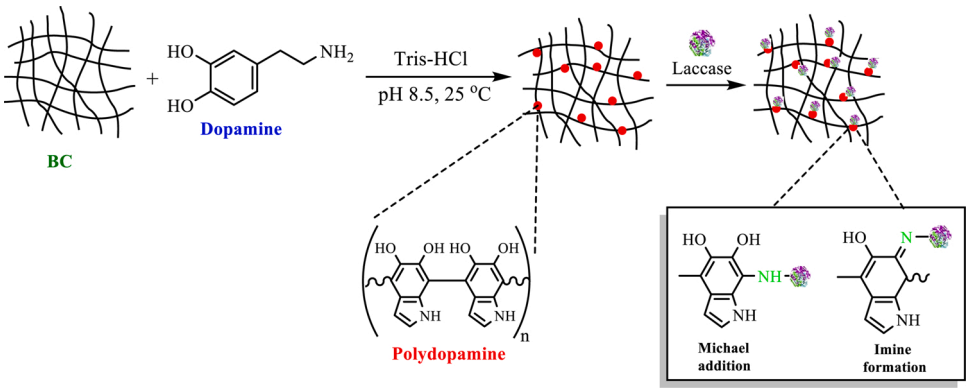

Fig. 3. Fabrication of polydopamine (PDA)/bacterial cellulose (BC) support for covalent immobilization of laccase (Zhai et al., 2019).

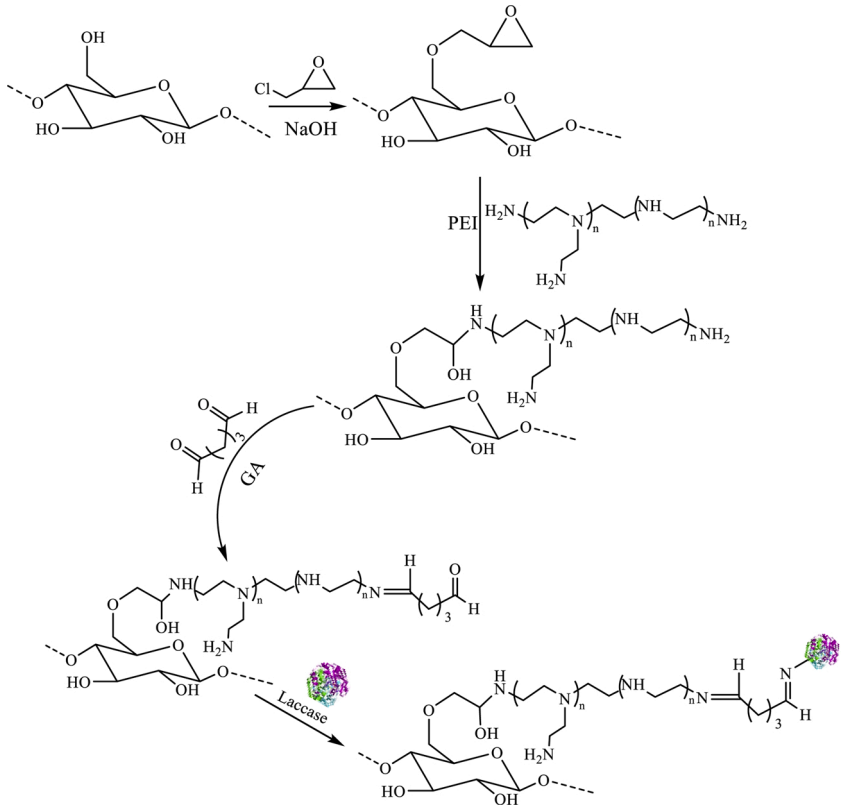

Fig. 4. Functionalization of CNFs with PEI to provide a support for immobilization of laccase (Bansal et al., 2018).

properties including large surface area, extensive pH and temperature range accompanied by enhanced thermal and storage stabilities (Asgher et al., 2017). It also degraded 100 % of five textile dyes (Sandal-fix Red C4BL, Sandal-fix Turq. Blue GWF, Sandal-fix Golden yellow CRL, Sandal-fix Black BR and Sandal-fix violet P4RN) within 4 h (Asgher et al., 2017). Using this biocatalyst, a dechlorination efficiency of ~ 61 % for Sandal-fix Red C4BL dye was achieved after 9 runs (Asgher et al., 2017). Alongside the reusability, the CS-immobilized laccase indicated higher thermal and pH (3.5–12) resistance than free counterpart (Zheng et al., 2016). Remarkably, the resulted biocatalyst could eliminate numerous dyes (Table 3) (Zheng et al., 2016). As it is shown, the efficiency of this biocatalyst for Acid Black 172 degradation was 1.2-fold greater than that of laccase in the free form (Table 3, entry 4) (Zheng et al., 2016). Similarly, *T. versicolor* laccase on the CS/GA macrobeads support with the aid of ABTS mediator could degrade 60 and 48 % of anthracene to the less toxic 9, 10-anthraquinone after 24 h using 1.25 % and 5% GA, respectively (Apriceno, Bucci, & Girelli, 2017). This immobilization strategy led to the higher thermal resistance of laccase and higher performance for the ABTS oxidation than free laccase. Moreover, the biocatalyst could recover simply by filtration or sedimentation.

By incorporating CeO_2 to GA-crosslinked CS microspheres, another biocatalyst was prepared by immobilizing laccase through covalent bonding and adsorption (Lin et al., 2015). The degradation activity of this biocatalyst was appraised for methyl orange II and methyl red dyes and furthermore compared with that of the CS microspheres-laccase and non-immobilized laccase. The maximum degradation for the above-mentioned dyes was attained in the presence of this biocatalyst with 92.6 % for methyl orange II and 83.3 % for methyl red. Also, further crosslinking of GA/CS beads by tripolyphosphate via electrostatic interactions improved the thermal stability of the support for covalent immobilization of laccase (Jeong & Choi, 2020). This hybrid biocatalyst showed effective performance in the degradation of tetracycline antibiotic to inactive oxidative compounds. In contrast to non-immobilized

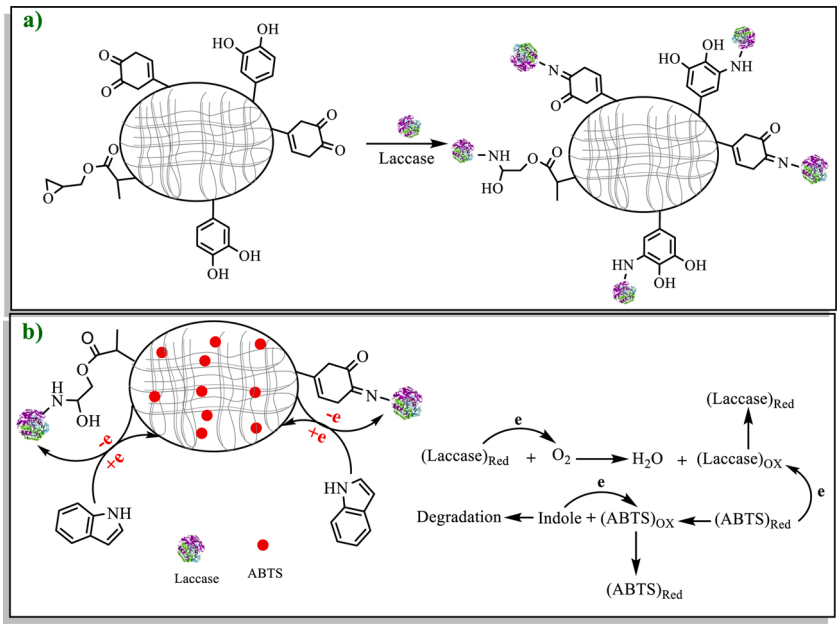

Fig. 5. a) Functionalization of the surface of cellulose beads with epoxides, CCs and quinones groups to yield a support for covalent immobilization of laccase (Gu et al., 2020); b) The proposed mechanism of indole degradation using laccase-ABTS system based on functionalized cellulose beads (Yaohua et al., 2018).

Table 3
The degradation of numerous dyes by laccase-loaded CS beads (Zheng et al., 2016).

Class of dye	Type of dye	Degradation (%)	
		Free laccase	Immobilized laccase
Anthraquinone	Reactive Brilliant Blue X-BR	47.2 ± 6.2	52.3 ± 6.8
Anthraquinone	RBBR	46.9 ± 7.8	48.2 ± 7.2
Azo	Congo Red	50.5 ± 7.6	54.2 ± 7.5
Azo (metal-complex)	Acid Black 172	56.3 ± 7.1	68.8 ± 6.7
Cyanine	Methylene Blue	17.2 ± 7.6	25.4 ± 7.5
Heterocycle	Neutral Red	44.9 ± 6.7	36.5 ± 6.8
Indigo	Indigo Blue	38.1 ± 6.5	45.1 ± 6.6
Nitroso (metal-complex)	Naphthol Green B	30.5 ± 7.4	37.2 ± 7.5
Phthalocyanine (metal-complex)	Direct Fast Blue FBL	48.8 ± 6.9	56.9 ± 6.8
Triphenylmethane	Crystal Violet	14.5 ± 7.7	20.8 ± 6.9

laccase, the resulted biocatalyst exposed high operative stability, since maintained about 60 % of the starting efficiency after 10 cycles. Immobilization also enhanced the thermostability: after 7 h treatment at 60 °C the biocatalyst maintained 80 % catalytic efficiency, while non-immobilized laccase kept lower than 50 % efficiency.

By replacing the highly toxic glutaraldehyde crosslinker with genipin, a naturally occurring crosslinking agent, the CS/genipin corsslinked beads were fabricated as stable supports for laccase (Ma, Meng, Cui, Si, & Dai, 2018). In comparison to free enzyme, the constructed biocatalyst showed a number of significant characteristics such as improved storage, thermal and pH stabilities, better degradation efficiency against Acid Black 172 dye, higher reusability capability with preserving over 55 % original activity after 11 successive runs.

Magnetization of the support has been applied as an appropriate idea to facilitate the separation efficiency of the immobilized laccases. Accordingly, various magnetic CS-based supports have been developed and loaded with laccases via chemical or physical tethering methods (Bayramoglu, Yilmaz, & Arica, 2010; Nadaroglu, Mosber, Gungor, Adıguzel, & Adiguzel, 2019; Ulu et al., 2020; Zhang et al., 2020).

T. versicolor laccase (Bayramoglu et al., 2010) and *Weissella viridescens* LB37 *laccase* (Nadaroglu et al., 2019) were covalently bound to the magnetic CS beads with the aid of epichlorohydrin and GA cross-linkers, respectively. The immobilization of laccases was achieved through the reaction of $–NH_2$ moieties on the enzyme with epoxy moieties (Bayramoglu et al., 2010) and aldehyde moieties (Nadaroglu et al., 2019) on the magnetic support surface. The biocatalyst with *T. versicolor laccase* exhibited the improvement of stability toward pH, storage and temperature as well as catalytic activity compared to free form (Bayramoglu et al., 2010). The capability of the biocatalyst was furthermore studied in the biodegradation of Reactive Blue 4 (RB-4) and Reactive Yellow 2 (RY-2) dyes in a batch reactor. Comparatively, the free laccase and the resulted biocatalyst could eliminate nearly 48 and 59 % RB-4 along with 71 and 82 % RY-2, respectively during 18 h treatment (Bayramoglu et al., 2010). On the other hand, the insolubilized *Weissella viridescens* LB37 laccase showed relative higher activity for ABTS oxidation than free counterpart, also 47 % of its relative activity was retained after ten runs in a batch system (Nadaroglu et al., 2019). Besides, it could be successfully utilized for the degradation of synthetic dyes including Direct blue 15, Evans blue, RB5 and Acid red 37 with yields of 94–96 %, during 60 min at 30 °C and pH 6. Similarly, Reactive Blue 171 and Acid Blue 74 textile dyes were efficiently decolorized by laccase-loaded amended Fe_3O_4 magnetic nanoparticles with thiolated CS (TCS) (Ulu et al., 2020). Disulfide formation between the thiol groups of TCS with cysteine moieties in laccase induced the covalent conjugation of the enzyme to the support. Compared to the non-immobilized enzyme, the developed magnetic biocatalyst (Fe_3O_4-TCS-Laccase) exhibited better resistance to temperature and pH as well as higher stability and reusability, which can be ascribed to the strong interactions between enzyme and the thiol moieties of TCS. Coating the CS on the surface of Fe_3O_4 nanoparticles and subsequent crosslinking and functionalization by GA produced a nano-support for covalent immobilization of laccase to eliminate the chloro-phenols from water solution (Zhang et al., 2020). After immobilization, the stability of the resulted biocatalyst against different parameters including temperature, storage, and pH was extensively enhanced. With the laccase loading of 90 mg/g, the resulted biocatalyst could degrade 91.4 % of 2,4-dichloro-phenol (2,

4-DCP) and 76 % of chloro-phenol (4-CP) after 12 h treatment. Besides the ease in separation, the biocatalyst displayed great reusability and maintained about 57–76 % of degradation efficiency after 10 runs.

Addition of inorganic fillers to the magnetic CS-based materials could further improve the thermal stability of the hybrid system. The magnetic CS-clay composite was also utilized as a supporting material for the covalent linkage of laccase by GA to provide a system for detoxification of phenol (Aydemir & Güler, 2015). Of note, the biocatalyst displayed more tolerance against pH and temperature variations accompanied by higher storage stability than non-immobilized counterpart. Thanks to the presence of magnetic Fe_3O_4, the biocatalyst system could be recycled by a magnetic field, also maintained about 75 % of its degradation efficacy after 10 successive runs. Using this biocatalyst, about 80 % of phenol was degraded at pH 5 and 25 °C after 4 h of treatment.

In another effort, halloysite nanotubes (HNTs) modified with supermagnetic Fe_3O_4 and coated with CS crosslinked by GA and utilized for immobilization of laccase (Kadam, Jang, Jee, Sung, & Lee, 2018). With the laccase loading of ~ 93 mg/g, the obtained biocatalyst showed better thermal and pH stability than the free enzyme along with dechlorination efficiency of 87 % for Direct Red 80 dye by the aid of ABTS. Additionally, it also displayed outstanding separation capability which is attributed to the magnetic property of carrier.

Likewise, Kadam and coworkers have recently reported the covalent attachment of laccase to HNTs which were initially amended with Fe_3O_4 nanoparticles and the CS beads (1%) and subsequently crosslinked with GA (Kadam et al., 2020). In this procedure, around 100 mg of laccase was loaded per gram of the magnetic support. The designed biocatalyst (HNTs-Fe_3O_4-CS (1%)-GA-Laccase) offered enhanced capability and stability toward different parameters including pH, temperature, reuse (preserved ~ 60 % of its starting activity after 10 runs) and storage time (kept 70 % of their initial activity after 30 days). The as-prepared biocatalyst was also able to eliminate about 41 %, 59 %, and 62 % of sulfamethoxazole (SMX), a pharmaceutical pollutant, using the redox mediators of ABTS, GUA, and SA, respectively. By the aid of an external magnet, the biocatalyst was effortlessly removed from the process and kept 57 % of SMX degradation efficiency after 6 consecutive runs.

Blending of CS with commercially available polymers and subsequent fabrication of nanofibers via electrospinning has also been proposed as a simple strategy to provide CS-based nanofiber supports with improved stabilities (Marysková et al., 2016; Xu, Zhou, Li, & Zhang, 2013). Covalently immobilized laccase to electrospun CS/PVA (PVA = polyvinyl alcohol) nanofibrous membranes using GA was employed in 2, 4-DCP degradation (Xu et al., 2013). This method led to the laccase loading of 853 mg/g on the carrier. The resulted biocatalyst displayed improved storage and thermal stabilities, concerned with the covalent association between the laccase and carrier, along with enhanced thermal stability which can be ascribed to the influence of the charge of the carrier. Using the optimal conditions (pH 6, 50 °C), the as-prepared biocatalyst eliminated about 88 % of 2,4-DCP and furthermore preserved 54 % of starting efficacy after 7 runs. Similarly, laccase was covalently connected to the modified polyamide 6/CS nanofibers with spacer hexamethylenediamine (HMD) or bovine serum albumin (BSA) and cross-linker GA (Marysková et al., 2016). The efficacy of the resulted biocatalysts (PA6/CS-GA-BSA-GA-Lac and PA6/CS-GA-HMD-GA-Lac) was investigated in the detoxification of endocrine-disrupting chemicals (EDCs) including 17-α-ethinylestradiol and bisphenol A. Notably, the biocatalysts showed almost similar activity and could degrade about 92 % of bisphenol A and 100 % of 17-α-ethinylestradiol. Findings clearly verified that the resulted biocatalysts were more active at degrading EDCs than free laccase. In addition, the PA6/CS-GA-HMD-GA-Lac maintained about 81 % of its starting efficacy after 14 days storage at 4 °C, whereas the PA6/CS-GA-BSA-GA-Lac was able to preserve only 55 % starting efficacy. On contrary, under identical conditions, the free laccase kept about 51 % of its initial efficacy. Therefore, it can be concluded that the immobilization also improved storage stability of laccase, especially in the presence of spacer HMD.

Beyond covalent immobilization strategy, physical immobilization approaches have also been applied for tethering the laccase on the CS structures. For instance, laccase was physically adsorbed on polyitaconic acid (pIA) grafted and Cu(II) chelated CS membranes (Fig. 6) and used for the degradation of RB5, Cibacron Blue F3GA (CB), and Methyl Orange (MO) dyes (Bayramoglu, Gursel, Yilmaz, & Arica, 2012). Electrostatic interaction between −COOH groups of pIA with $-NH_2$ groups of laccase led to the tight adhesion of the laccase to the support material. Employing the mediator acetosyringone, the resulting biocatalyst was more effective for the removal of MO (87 %) in comparison to CB (69 %) and RB5 (43 %) dyes. As well, the thermal and pH stability of laccase was improved after immobilization, verifying that the synthesized supporting material was appropriate for insolubilization. Moreover, the free laccase lost all of its efficiency during 5 weeks of storage at 4 °C, while the developed biocatalyst lost only 37 % of its original efficiency during 8 weeks. Accordingly, the insolubilization also enhanced storage stability of enzyme.

In another study, laccase was adsorbed on Cu(II)-chelated CS nanoparticles and as a biocatalyst for phenol detoxification showed higher performance than free laccase (Alver & Metin, 2017). The enzyme immobilization was performed by fabrication of CS-graft-poly(glycidyl methacrylate) (CS-g-PGMA) nanoparticles and further functionalizaion with PEI through the reaction of epoxide groups of PGMA with amino groups of PEI. The loading of Cu(II) into the resulted CS-g-PGMA/PEI nanoparticles facilitated the attachment of laccase by physical adsorption through chelating phenomenon. Indeed, chelating sites in enzymes such as thiol, indolyl, amine and imidazole groups can potential sites for the chelation with metal centers (Bayramoglu, Senel, & Arica, 2006; Zou et al., 2011). With laccase loading of 66 ± 2.5 mg/g of support, the resulted biocatalyst presented broader temperature and pH range, higher stability and reusability as compared to free enzyme. Using ABTS, this biocatalyst also exhibited outstanding performance for the phenol removal. Significantly, the degradation percentage of phenol was greater than 96 %.

5.3. Alginate

Alginates, linear anionic polysaccharides found in algae, consist of alternating blocks of (1, 4)-linked α-L-guluronate (G) and β-D-mannuronate (M) residues (Pawar & Edgar, 2012; Yang, Xie, & He, 2011). They can form soluble salts with monovalent metal ions while with divalent (other than Mg^{2+}) or multivalent metal ions generate gels (Tønnesen & Karlsen, 2002). Nowadays, the immobilization of biomaterials onto alginates because of their advantages such as high natural abundance, low cost, non-toxicity, biocompatibility, biodegradability, and chelating ability has received significant importance (Zia, Zia, Zuber, Rehman, & Ahmad, 2015).

In a simple approach, laccase was physically entrapped in Cu(II)-alginate beads and utilized for dye degradation (Teerapatsakul, Bucke, Parra, Keshavarz, & Chitradon, 2008; Teerapatsakul, Parra, Keshavarz, & Chitradon, 2017). Almost 100 % of Indigo Carmine dye was eliminated by the entrapped enzyme in Cu-alginate beads containing 3.6 % w/v alginate and 0.15 M $CuSO_4$ (Teerapatsakul et al., 2008). Importantly, the biocatalyst afford to degrade 96 % of Indigo Carmine after 9 successive runs during 12 days, which was 5 times greater than acquired by the free laccase. When compared, the entrapped laccase demonstrated much greater pH (up to 10.0) and temperature (up to 55 °C) resistance than free enzyme (Teerapatsakul et al., 2017). It was also efficient in the removal of numerous dye classes under non-buffered condition (see Table 4) (Teerapatsakul et al., 2017). Employing this system lowered costs owing to its operation in tap water. In addition, the immobilized laccase revealed supreme reusability because it was able to the carry out fourteen cycles of complete Indigo Carmine degradation which reveals its potential to be used in high scale industrial application.

In a similar strategy, entrapped laccase in Ca(II)-alginate beads was

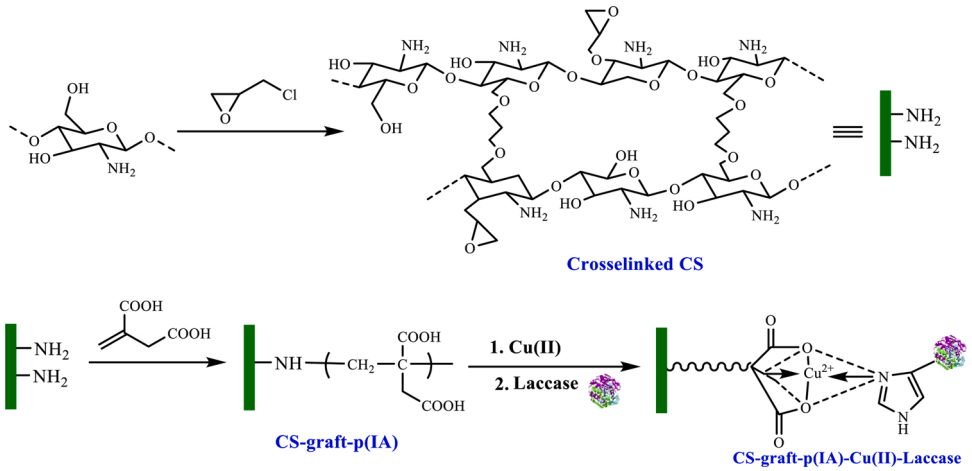

Fig. 6. General strategy for grafting of poly(itaconic acid) on epichlorohydrin-crosslinked CS along followed by subsequent immobilization of laccase (Bayramoglu et al., 2012).

Table 4
The removal of numerous dyes using the immobilized laccase in Cu(II)-alginate beads (Teerapatsakul et al., 2017).

Class of dye	Type of dye	Degradation[a] (%)
Indigoid dye	Indigo Carmine	100[b]
Anthraquinone dye	RBBR	100[b]
	Bromophenol Blue	64.4
Triphenylmethane dye	Crystal Violet	48.5
	Malachite Green (MG)	82
	Congo Red	64
Azo dye	Direct Blue 15	54
	Direct Red 23	22

[a] Dye degradation in 12 h.
[b] Dye degradation in 2 h.

employed for the treatment of different textile dyes including Bismark Brown R, RBBR, Reactive Black 5 (RB5) and Lanaset Grey G (Daâssi, Rodríguez-Couto, Nasri, & Mechichi, 2014). Immobilization onto Ca (II)-alginate beads considerably amended the pH and temperature stability of laccase. The free and immobilized laccase displayed a low efficiency in the degradation of aforementioned dyes (except RBBR) without redox mediators, whereas they were more efficient in the existence of the HBT mediator. Meanwhile, the alginate-immobilized laccase showed remarkable operative stability with retention of 70 % starting activity after four runs except for Bismark Brown R (~ 51 %).

Comparatively, Cu(II)-alginate exhibited higher binding affinity toward laccase than Ca(II)-alginate and the efficiency of the immobilized laccase on Cu(II)-alginate was higher than that for Ca(II)-alginate (Niladevi & Prema, 2008). Laccase on Cu(II)-alginate support showed notable degrading efficiency towards different phenolic compounds including gallic acid, tannic acid, ferulic acid, resorcinol, GUA, CC, vanillic acid and pyrogallol in packed bed bioreactor (Niladevi & Prema, 2008). Practically, almost 95 % of these compounds were oxidized during 30 min. However, the biocatalyst presented reusability with only 50 % original efficiency after 8 consecutive runs. Similarly, entrapped laccase onto the Ba(II)-alginate at concentration 0.57 U/g-alginate could eliminate 94 % of acetaminophen, a pharmaceutical pollutant, during 4 h at 35 °C and pH 7 (Ratanapongleka & Punbut, 2018). Compared to free enzyme, the entrapped laccase also demonstrated enhancement on stability and reusability, as it was capable of eliminating over 70 % of this pollutant after five catalytic runs.

An easily separable core-shell magnetic laccase/Cu(II)-alginate hybrid system was developed by incorporating iron oxides (nFe$_2$O$_3$) in the enzyme/support hybrid system (Le et al., 2016). While magnetic nFe$_2$O$_3$ act as core, the laccase/Cu(II)-alginate remained as the shell in the catalytic system. The encapsulated laccase presented high enzyme loading coupled with profound stability in water and acetate buffer. Moreover, this magnetic biocatalyst degraded about 90 % of triclosan, a pharmaceutical compound, after 8 h. It also eliminated RBBR dye (~ 54–75 %) after 4 h. Owing to magnetic core (nFe$_2$O$_3$), this biocatalyst was effortlessly recovered by a magnet and reused, resulting in reduction of the operational cost.

The co-immobilized laccase and ABTS mediator onto layered double hydroxide (LDH)/alginate beads were applied for the oxidative degradation of Malachite Green (MG) dye (Fig. 7) (Huang et al., 2018). Using co-precipitation method, ABTS molecules were initially intercalated into Zn-Cr LDH materials. Then, laccase was attached on the ABTS-LDH through physical adsorption. Finally, alginate beads were utilized to encapsulate the obtained hybrid materials. The dye removal efficiency of the immobilized laccase-mediator system (Im-LMS) (92 % in 120 min) and the non-immobilized laccase-mediator system (90 % in 90 min) were almost the same. As well, the Im-LMS attained 79 % degradation of MG after 9 repeated runs and the oxidation efficacy remained unchanged after ten days.

Dual-functionalization of alginate by grafting polymerization with poly(glycidyl methacrylate) and coupling of the —COOH groups with dopamine afford a proper derivative for co-immobilization of ABTS and laccase onto the Ca-alginate beads (Fig. 8) (Xue et al., 2020). The resulting laccase-mediator system unveiled superb efficacy for the biodegradation of acridine, a nitrogenous heterocycle employed for the synthesis of dyes and medicines. Of note, the biocatalyst could degrade 100 % of acridine (30 mg/L) at 30 °C and pH 6 after 8 h, while free laccase degraded acridine with very low yield. As well, the biocatalyst showed pronounced reusability by maintaining 93.4 % acridine degradation efficiency after four runs.

5.4. Kinetics parameters of immobilized laccases

Table 5 shows the values of the kinetic parameters, Michaelise-Menten constant (K_m) and maximum reaction rate (V_{max}), of free and immobilized laccases on various polysaccharide supports for the oxidation of different substrates. These kinetic parameters are calculated from the Lineweaver-Burk plot of 1/V versus 1/S as follows:

$$1/V = (Km/Vmax)(1/S) + 1/Vmax$$

V_{max} is the highest reaction rate attained by the biocatalyst and K_m is the substrate concentration at which the reaction rate is half of the V_{max}. It is to be noted that K_m is used as an inverse indicator of the affinity of an enzyme for a substrate. A higher value of K_m indicates that the enzyme has a lower affinity for the substrate. As it is shown in Table 5,

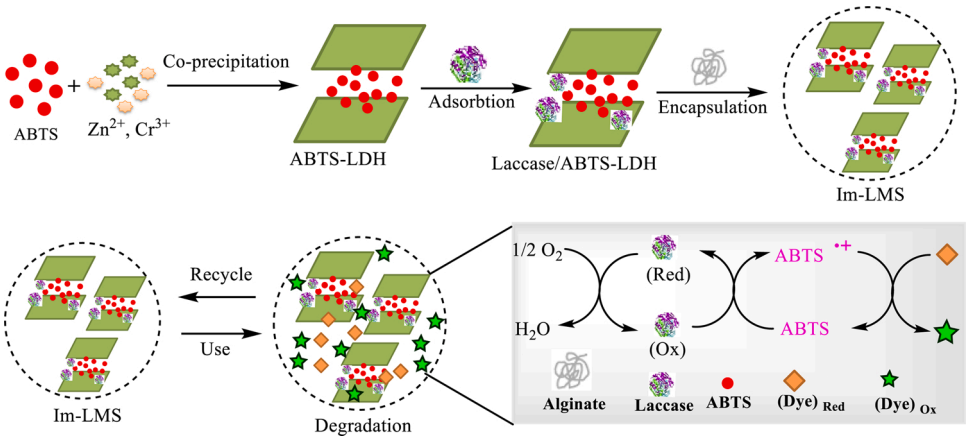

Fig. 7. A Schematic of Im-LMS synthesis and MG oxidation (Huang et al., 2018).

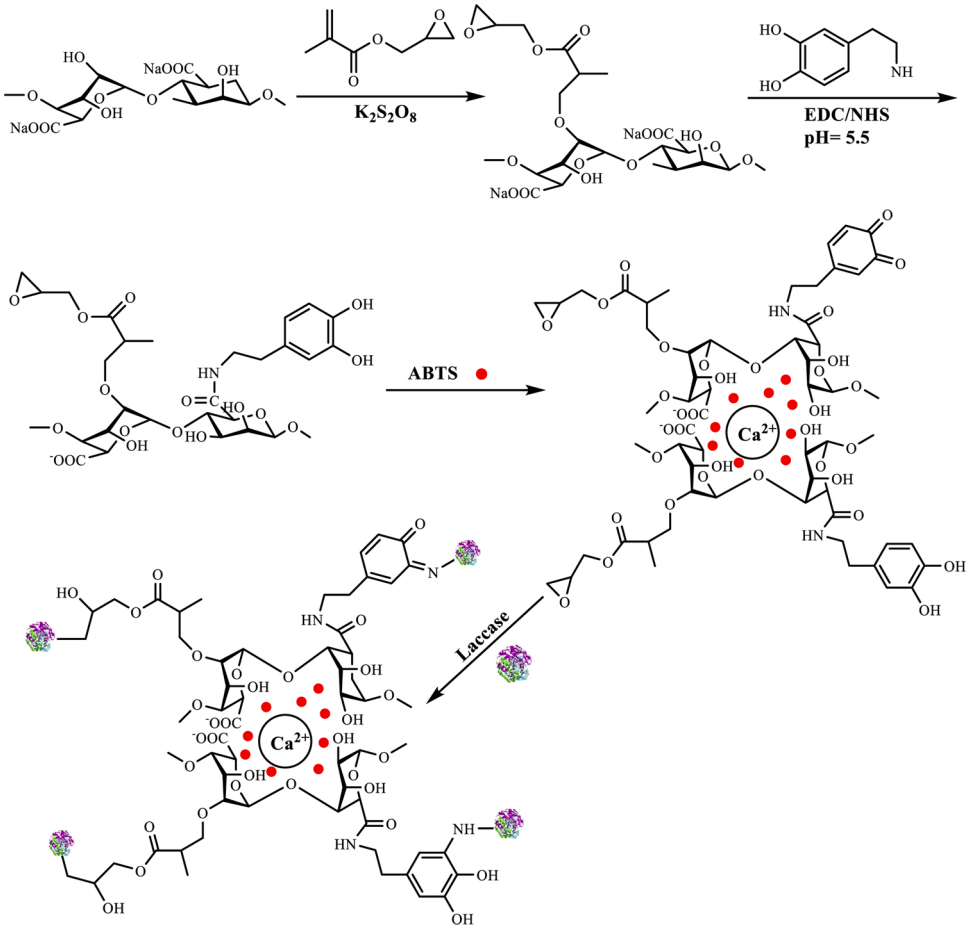

Fig. 8. Dual-functionalization of alginate with epoxide and dopamine groups and its application as proper support for co-immobilization of ABTS and laccase (Xue et al., 2020).

immobilization process can increase or decrease the amount of K_m and V_{max}. The higher K_m after immobilization indicates that immobilized enzyme has a lower binding affinity towards the substrate, attributed to loss of enzyme flexibility for substrate binding, enzyme conformational changes after immobilization and or lower accessibility of the substrate to the active sites of the immobilized enzyme caused by the increased diffusion limitations (Ratanapongleka & Punbut, 2018; Rekuć, Bryjak, Szymańska, & Jarzębski, 2009).

5.5. Trends on laccase immobilization over the last few decades

During last two decades significant progress have been achieved regarding the immobilization of laccases. Most of the old strategies are including the pre-functionalization of the inorganic supported by silylation with 3-aminorpopyltriethoxysilane (APTES) to insert amino groups on the support surface and then crosslinking of the laccase to the surface of the supports using glutaraldehyde (Davis & Burns, 1992; Sarkar, Leonowicz, & Bollag, 1989). However, in last two decades diverse types of polysaccharides with various functional groups as the

Table 5
Kinetic parameters for the oxidation of substrates by free and immobilized laccases.

Laccase	Substrate	K_m (μM)	V_{max} (U/mg)	Ref.
Pleurotus florida NCIM 1243 laccase	ABTS	161.1	2.93	(Sathishkumar et al., 2014)
Pleurotus florida NCIM 1243 laccase immobilized onto dialdehyde CNFs		343.1	2.76	
Free laccase	ABTS	44	0.49	(Bansal et al., 2018)
Laccase immobilized onto CNFs/PEI/GA		37	0.47	
T. versicolor laccase	ABTS	28.3	379.5	(Lin et al., 2015)
T. versicolor laccase immobilized onto GA-crosslinked CS/CeO_2 microspheres		101.1	225.8	
Trametes trogii laccase	ABTS	140.3	175.4	(Ulu et al., 2020)
Trametes trogii laccase immobilized onto Fe_3O_4-TCS composite		45.4	909.1	
T. versicolor laccase	ABTS	297	26.9	(Aydemir & Güler, 2015)
T. versicolor laccase immobilized onto magnetic CS-clay/GA		410	12.1	
T. versicolor laccase	Syringaldazine	9.4	21.7	(Bayramoglu et al., 2010)
T. versicolor laccase immobilized onto epoxy-functionalized magnetic CS		19.7	15.6	
T. versicolor laccase	Syringaldazine	19.7	20.4	(Bayramoglu et al., 2012)
T. versicolor laccase immobilized onto pIA grafted and Cu (II) chelated CS membranes		41. 6	16.5	
T. versicolor laccase	Syringaldazine	55	0.2	(Alver & Metin, 2017)
T. versicolor laccase immobilized onto CS/PGMA/PEI/Cu (II)		70	0.14	
T. versicolor laccase	Tetracycline	307.6 ± 29	32.2 ± 1.3	(Jeong & Choi, 2020)
T. versicolor laccase immobilized onto CS/TPP/GA		278.4 ± 38	76.3 ± 4.3	
Lentinus polychrous laccase	Acetaminophen	203.6	15.8	(Ratanapongleka & Punbut, 2018)
Lentinus polychrous laccase immobilized onto Ba(II)-alginate		98.9	1.4	

Abbreviations: cellulose nanofibers (CNFs), polyethylene imine (PEI), glutaraldehyde (GA), chitosan (CS), thiolated chitosan (TCS), polyitaconic acid (pIA), poly(glycidyl methacrylate) (PGMA), tripolyphosphate (TPP).

alternative supports have been applied directly for covalent conjugation to the laccase in the presence of a proper coupling agent. Another important progress is about co-immobilization of laccase and mediator which increase the activity, efficiency, and the range of laccase substrates. Finally, utilizing composite supports can be considered as another progress in laccase immobilization. For instance, incorporation of magnetic nanofillers to the polysaccharide-based supports was introduced during last two decades that combines high loading of the enzyme on the support with easy catalyst separation.

6. Conclusions and outlook

Laccases are employed as green catalysts in diverse oxidative reactions as well as in bioremediation of industrial effluents. To develop the performance of laccases and to facilitate their reuse, various immobilization strategies and supporting materials have been developed. Polysaccharides, as one of three major classes of natural polymers, have been increasingly employed as support carriers for laccases immobilization due to the following reasons: (1) they occur abundantly in nature and the methods of their isolation from natural sources are well-known; (2) they display promising features like easy availability, low cost, non-toxicity, biocompatibility, biodegradability, flexibility, and high mechanical strength; (3) they have partial negative influence on enzyme conformation and can bind to enzymes in a reversible and irreversible way because of the existence of plentiful functional groups along polymer backbones; (4) they can be used in nonaqueous environments since native enzymes only show their activity in aqueous media; and (5) chemo-, regio- and stereo-selectivity properties of the enzymes can be preserved after immobilization onto polysaccharides. These can increase the yield of final product and reverse any unwanted reaction.

Evaluating the feasibility and effectiveness of employing polysaccharides such as cellulose, chitosan, alginate and their composites as supporting materials for the immobilization of laccases demonstrate amended stability, enhanced catalytic activity along with improved separation and recyclability. Despite the aforementioned properties and advantages, there are the following restrictions in employing these polysaccharides for the laccase insolubilization:

- Polysaccharides generally have small surface area, resulting in loading a low amount of enzyme. However, the construction of nanofibers along with employment of inorganic/organic materials with large surface area into the polysaccharide matrix can enhance the amount of loaded laccase.
- When polysaccharides are hydrophilic, the separation procedure will be challenging. For instance, chitosan is soluble in acidic media and therefore cannot be used as support under these conditions. To overcome this issue, chemical modifications and crosslinking have been proposed. Crosslinking boosts the stability of chitosan in aqueous medium in the vast range of pHs.

On the other hand, it is noteworthy that magnetization of the supports facilitates the separation efficiency of the laccase/support system. The laccase/polysaccharide systems disclose the excellent efficacy in both biosensing and environmental applications (e.g. the degradation of dyes and xenobiotics).

Authorship agreement

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