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Review article

Applications and immobilization strategies of the copper-centred laccase enzyme; a review

Hilda Dinah Kyomuhimbo, Hendrik G. Brink *

Water Utilisation and Environmental Engineering Division, Department of Chemical Engineering, University of Pretoria, South Africa

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ABSTRACT

Laccase is a multi-copper enzyme widely expressed in fungi, higher plants, and bacteria which facilitates the direct reduction of molecular oxygen to water (without hydrogen peroxide production) accompanied by the oxidation of an electron donor. Laccase has attracted attention in biotechnological applications due to its non-specificity and use of molecular oxygen as secondary substrate. This review discusses different applications of laccase in various sectors of food, paper and pulp, waste water treatment, pharmaceuticals, sensors, and fuel cells. Despite the many advantages of laccase, challenges such as high cost due to its non-reusability, instability in harsh environmental conditions, and proteolysis are often encountered in its application. One of the approaches used to minimize these challenges is immobilization. The various methods used to immobilize laccase and the different supports used are further extensively discussed in this review.

1. Introduction

Laccase (EC 1.10.3:2) is a multi-copper enzyme that was first discovered in the discharge of *Rhus Verniciflua* by Yoshida in 1883 and later characterized as a fungal enzyme (metal containing oxidase) by Bertrand in 1985 [1,2]. The enzyme has been widely identified in fungi and higher plants, however it was only recently (1993) isolated in bacteria with the first bacterial laccase identified from *Azospirillum lipoferum* [3, 4]. All the laccases described to date are glycoproteins (monomeric, dimeric, and tetrameric) that show a great deal of divergence [5]. Although the catalytic site is conserved, diversity is observed in the rest of the molecule structure and sugar moiety [6].

Laccase catalyses direct reduction of molecular oxygen to water, through a one electron oxidation of aromatic substrates without formation of hydrogen peroxide intermediate, by one electron abstraction [7, 8, 9]. Molecules containing an aromatic ring substituted with an electron withdrawing groups such as phenols, cresols, chlorophenols, aryldiamines, aromatic amines, and polymethoxybenzenes all undergo oxidation in the presence of laccase [10, 11]. Upon oxidation, free cationic radicals are formed that further undergo catalysed oxidation to form quinones or non-enzymatic reactions such as hydration and polymerization occur to form high molecular weight insoluble components such as dimers, oligomers, or polymers [12, 13, 14, 15]. Thus, laccase shows broad specificity of its substrates and has gained popularity in detoxification processes of aquatic and xenobiotics, industrial effluents, and biotechnological industrial applications [16, 17].

Physical methods such as circular dichroism and Electron paramagnetic resonance (EPR) have been used to study laccase structure.

* Corresponding author.
E-mail address: deon.brink@up.ac.za (H.G. Brink).

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It has been observed that the metallic centres are relatively stable but the secondary structures differ due to varying amino acid sequence and/or composition of the carbohydrate moiety [6]. Due to the diversity in the enzyme secondary structures, the substrates oxidized by laccase vary from one laccase to another ranging from inorganic or organic metal complexes, ferrocyanides, anilines, benzenethiols, and phenols to other redox inorganic, organic, or biological compounds [18, 19]. For example Xu *et al.* [20], demonstrated that the catalytic properties of laccase from the fungi *Rhizoctonia* sp. and *Myceliophthora thermophile* as well their inhibition were altered by type 1 copper site directed mutagenesis.

Laccase catalytic reactions occur in ambient temperatures, use molecular oxygen as the secondary substrate reducing it by a four electron mediator-less mechanism, do not require any other cofactors and only water is produced as the by-product [21, 22]. Due to their extracellular nature, laccases can tolerate high pollutant concentrations in substrates [23]. These advantages and the ability to produce laccase by simple and inexpensive methods make it desirable in various industrial and environmental applications including organic synthesis, degradation of toxic organic compounds, biosensors, and immunoassays [24, 25, 26]. As a result, it has attracted research interest in order to understand its mechanism and obtain a scientific basis for its employment in biotechnological applications [27].

However, with all the advantages of using laccase, it has its own shortcomings including very low stability in harsh conditions and non-recoverability which limits its application at an industrial scale [28]. Recently, attention has been placed on the enzyme immobilization in a confined space or on a support to improve and optimize its performance for commercial use as a biocatalyst and facilitate its reuse [28, 29]. This review explores the various sources of laccase in nature, its structures and proposed mechanisms of catalytic activity, its roles in nature, and its industrial and environmental applications. The immobilization techniques that have been employed over the years to improve its stability and reusability as well as the various immobilization supports are also extensively discussed. The various sections discussed in this review about laccase enzyme are schematically represented in Figure 1.

2. Structure and reaction mechanism of laccase enzyme

Laccase is a blue oxidase glycoprotein (monomeric, dimeric, or tetrameric) with an amino acid chain over 500 amino acids and carbohydrate moiety of 10–45% of the protein molecule by weight [28, 30]. Different carbohydrates including glucose, hexose amine,

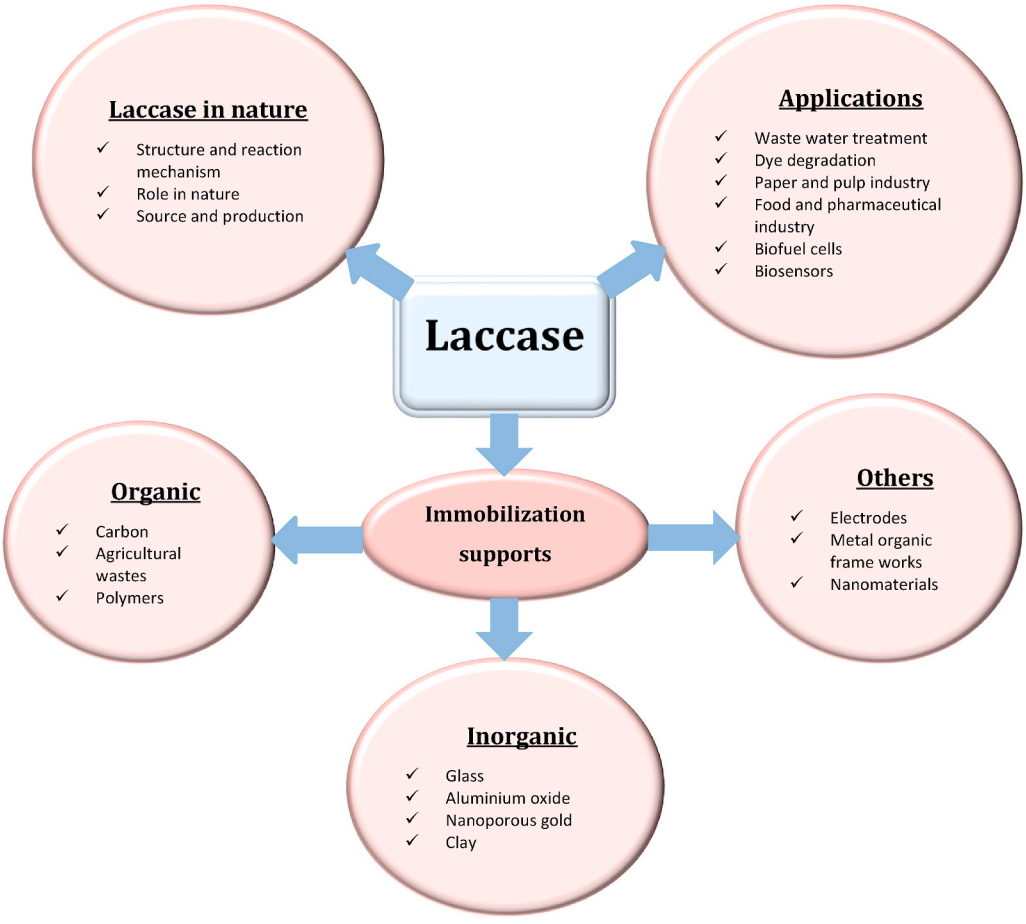

Figure 1. A schematic representation of what is covered in this review about laccase enzyme.

mannose, galactose, fructose, xylose and arabinose make up the carbohydrate moiety (glycans), are bound to the polypeptide of the enzyme through an N-linkage and vary depending on the source of the enzyme [31, 32]. The binding of the carbohydrate moieties to the enzyme defines the structure of the enzyme through a specific folding which provides protection against proteolytic and elevated temperature degradations [32].

More than one laccase are often identified in a single fungus with different molecular weights, pH optima, sugar moiety, and substrate specificity [6]. For example, Kim *et al.* [33] discovered a second extracellular laccase from *Crypthonectria parasitica* by deleting laccase 1 using recombinant DNA techniques and growing the mutant strain in tannic acid. Wahleithner *et al.* [34] identified four distinct laccase genes in the fungus *Rhizoctonia solani* and three of which had been expressed in another fungus *Aspergillus oryzae*.

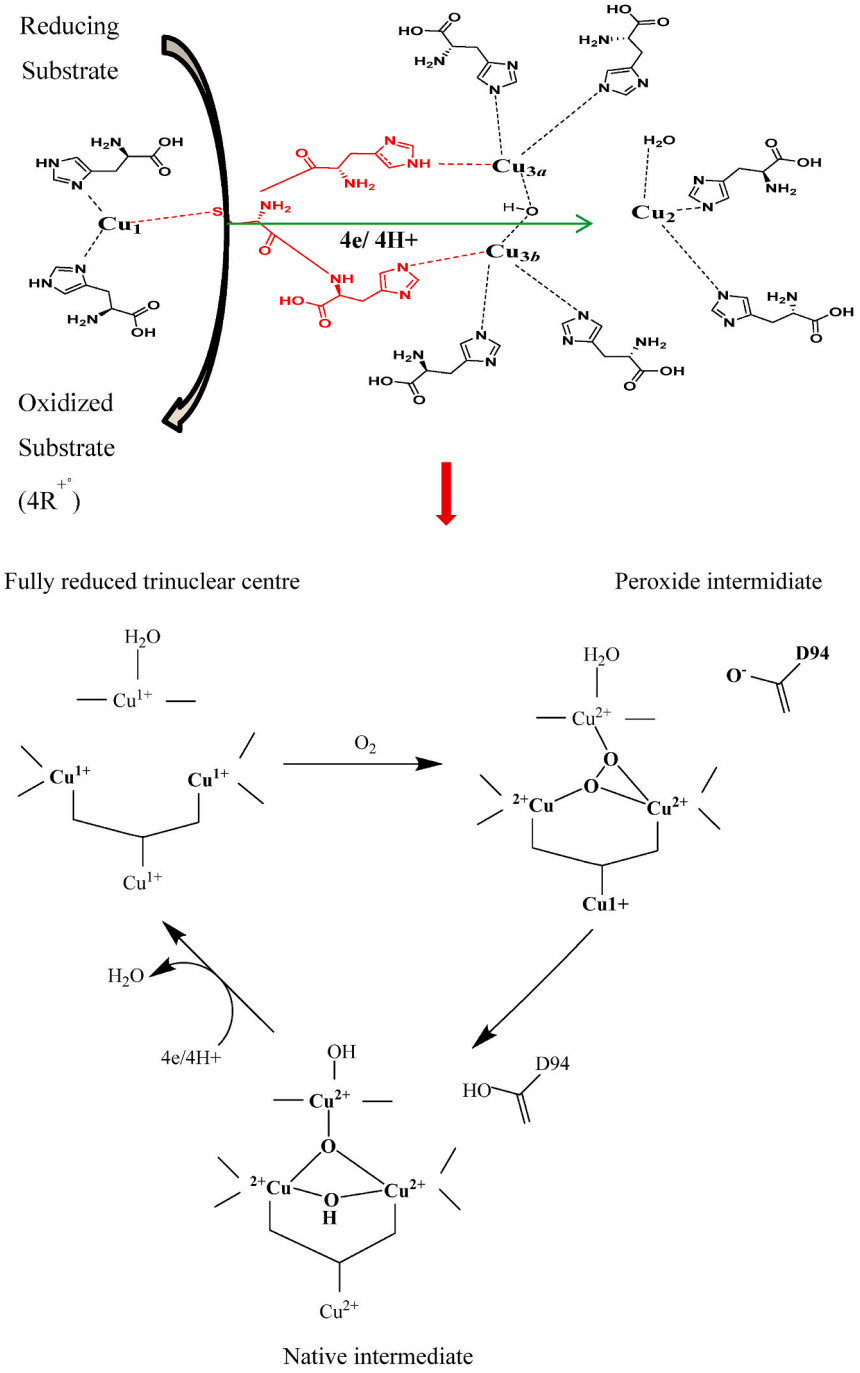

Figure 2. A schematic representation of the different amino acids that coordinate with the copper at the laccase site and the reaction mechanism for the laccase catalytic activity.

Marbach *et al.* [35], identified two molecular forms of laccase induced by different phenolics from *Botrytis cinera* with different sugar content, isoelectric focusing pattern, amino acid composition, and molecular weight. The enzyme induced by grape juice had a molecular weight of 38,000 and 80% sugar while that induced by gallic acid had molecular weight of 36,000 and 70% sugar. Fukushima and Kirk [36] identified two laccases from the fungus *Ceriporiopsis subvermispora* with two isoelectric points (3.4 and 4.8), molecular masses of 71 and 68 kDa, half-lives of 120 and 50 min at 60 °C, and carbohydrate content of 15 and 10%, respectively.

The active holoenzyme form of laccase consists of four copper atoms that are classified into three types depending on the features of their surroundings, their accessibility to solvents, and characteristic electron paramagnetic resonance (EPR) signals [37, 38]. The copper atoms are classified into type 1 (blue), type 2 (normal) and type 3 (coupled binuclear) copper with two copper atoms belonging to the type 3 site [30, 39]. Type 1 and type 2 copper have strong electronic adsorption and well characterized EPR signals and type 3 copper atoms are antiferromagnetically coupled through a binding ligand which makes the EPR signal undetectable but give a weak absorbance in the near UV region at 330 nm [30, 40]. Type 1 copper is responsible for the intense blue colour of the enzyme due to a ligand-to-metal charge transfer absorption of the copper cysteine-bond [41, 42]. It is further available to interact freely with solvents (water inclusive), can be removed from the enzyme molecule by various copper complexes, and can displaced by mercury or cobalt with a great loss in activity [30]. Type 2 copper coordinates two Histidine (His)-N and an oxygen atom as OH^- while each copper of type 3 coordinates three His residues [43, 44]. The type 1 and type 2/3 copper are bridged by a His-cysteine (Cys)-His tripeptide bridge that acts as an intramolecular electron transfer highway [45].

Substrates attach to the binding site of laccase by hydrophobic interaction and all the copper ions are presumed to be involved in the catalytic mechanism [46, 47]. As demonstrated in Figure 2, Type 1 and 2 are presumed to participate in electron capture and transfer while type 2 and 3 are involved in binding with oxygen or oxygen uptake [38, 48]. The catalysis process of laccase comprises of type 1 copper reduction by the reducing substrate, internal electron transfer from type 1 to type 2 and 3 trinuclear structure, and ultimate activation and reduction of oxygen to water at the type 2 and 3 copper trinuclear structure (Figure 2) [42, 49, 50]. The O_2 molecule binds to the trinuclear cluster for asymmetric activation and it is presumed that the O_2 binding compartment restricts access of other oxidizing agents other than oxygen [30, 49].

Laccase-like enzymes that lack typical absorption at 600 nm have been identified. For example, Palmeri *et al.* [41], reported a white laccase from *P. ostreatus* that contained one copper, two zinc, and one iron atoms per mole of enzyme and did not have an absorbance at 600 nm hence the classification of white laccase. The enzyme is considered a laccase due to its identical structure to other laccases and the fact that it uses oxygen as the oxidizing substrate [51]. Leotievsky *et al.* [52], identified yellow laccase from solid-state culture of ligninolytic fungi as a result of binding lignin-derived molecules by the enzyme protein. The yellow laccases show high homology with the blue laccases when their N-terminal amino acids sequences are compared. They have similar copper content to the typical blue laccase but don't maintain their copper centres in the oxidized state under anaerobic conditions, that is, the copper in the yellow laccase appears in the reduced form. The change in this enzyme property is attributed to binding of low molecular mass phenolic material arising from lignin degradation or heterogeneity induced by glycosylation [51].

Laccases are inhibited by metal ions such as Hg^{2+}, Fe^{2+}, Ag^{+}, Li^{+} and Pb^{2+} [53, 54, 55], humic acid [56], cationic quaternary ammonium detergents and small anions like halides [57, 58, 59], azide [60, 61], and hydroxyl [62]. The inhibition process involves amino acid residue modification, copper chelation or conformational changes, and binding of small anions on type 2 and 3 copper hence interrupting internal electron transfer [58, 59]. Enzyme inhibition due to organic solvents occurs as a result of hydrophobic interactions between the solvent and non-polar groups of laccase and substitution of hydration water within the enzyme with the organic solvent. This happens because the binding site of the organic substrate in the enzyme is close to the surface of enzyme globule hence readily accessible for the solvent [63, 64]. Halides bind to the type 2 and 3 copper of the enzyme and inhibit electron transfer from type 1 copper to the trinuclear structure thus leading to enzyme catalytic decay [65]. Metal ions competitively inhibit laccase due to their strong affinity to thiol groups in laccase but have no impact on the enzyme active centre [66]. Interestingly, the presence of divalent metal ions has shown to improve laccase activity due to their competition with Cu^{2+} in the electron transport system which improves the enzyme-substrate relationship [67].

With the wide range of substrates that can be oxidized by laccase, addition of mediators such as 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ABTS [68, 69], N-hydroxybenzotriazole (HOBT) [70], and 2,2,5,5-tetramethyl-4-piperidin-1-oxyl radical (TEMPO) [71, 72] to a specific laccase enzyme can further increase the substrate range to include non-phenolic compounds. Mediators are low-molecular-weight organic compounds that are oxidized by laccase to form highly active cation radicals which oxidize the non-phenolic compounds that are usually resistant or impervious to laccase oxidation through the normal shuttling of electrons from the substrate to the enzyme [73, 74, 75]. For example, Peralta-Zamora *et al.* [76], used HOBT as a mediator in the decolourisation of azo and indigo dyes (usually not laccase substrates) using free laccase and laccase immobilized on silica modified with imidazole. No decolourisation was detected in the absence of HOBT but achieved 30% and 45% decolourisation efficiency for free and immobilized laccase respectively within 30 min after adding 0.4 mL of HOBT. Dodor *et al.* [77], used *T. versicolor* laccase immobilized on kaolinite for the catalytic oxidation of anthracene and benzo [a]pyrene in the presence of ABTS as a mediator. Between 17% and 19% oxidation were achieved without a mediator, but after addition of ABTS the oxidation efficiency increased to 80% and 85% for anthracene and benzo [a]pyrene respectively. Gu *et al.* [78], covalently immobilized laccase on ABTS-encapsulated cellulose beads for indole degradation. While free laccase hardly degrades indole, the biocatalyst achieved a degradation rate of up to 99.7%. Reyes *et al.* [79], demonstrated that the number of decolourised dyes by laccase increased from 13 to 26 out of 38 dyes when 1 mM HOBT was added as a mediator and increased rate of decolourisation was observed. Laccase immobilized on concanavalin A-activated Fe_3O_4 nanoparticles demonstrated an exponential increase in the rate of removal of sulfonamide antibiotics when syringic acid was used as a mediator [80].

However, the benefits of mediators are sometimes negated by their disadvantages such as toxicity, cost, and the ability of radical

species from mediator compounds to undergo chemical reactions with aromatic side-chains of laccase thereby inactivating the enzyme [81, 82]. For example, Skoronski *et al.* [83], observed that it was impossible to reuse *Aspergillus* sp. laccase immobilized on chitosan during syringaldazine bioconversion. More so, the approach used in introducing a mediator has an impact on the performance of the biocatalyst. For example, when laccase and acetylacetone (mediator) were co-immobilized through initiated polymerization into a hydrogel, the biocatalyst displayed a high substrate conversion of malachite green as compared to the sole immobilized laccase and immobilized laccase with an external mediator [84]. Therefore, factors such as good laccase substrate with high redox potential intermediate and with stable reduced and oxidized forms that do not inhibit enzymatic reaction should be considered when selecting a redox mediator [85].

3. Role of laccase in nature

Due to the large amounts of laccase produced by lignin degrading (wood rotting) fungi, it has been presumed that the main role of laccase in nature is the polymerization and degradation of lignin [63, 86]. Laccase mediated degradation of lignin starts with the loss of an electron from the phenolic hydroxyl groups of lignin to produce phenoxy radicals which spontaneously re-organizes to aid either α-carbon oxidation or C_α-C_β bond cleavage of alkyl side chains of the polymer to produce low molecular weight and/or polymeric products due to polymerizing activity of the enzyme [30, 87]. Fungal laccases are argued to be involved in the regeneration of tobacco protoplasts [88], lignification of cell walls [6, 89], morphogenesis of rhizomorphs [37], sclerotization and melanogenesis processes in insects [90, 91, 92], fungal virulence, sporulation and plant pathogenesis [49, 93].

Laccase has been suggested to play a role in humus synthesis. Laccase catalyses the cleavage of lignin and non-lignin macromolecules into their monomeric structural units which react with other compounds to form humic substances of varying complexity [49, 94]. Additionally, laccase catalyzes coupling reactions between phenolic compounds and humic acids leading to the binding of pollutants on organic matter of soil hence reducing their bioavailability and toxicity [11, 32]. Because of this, laccases have been investigated for applications in detoxification of pollutants in soil environments. Polluted soils can be cleaned up by laccase-mediated incorporation of pollutants into humus through catalytic coupling of xenobiotic compounds and derivatives to humic substance or humic like compounds [49].

4. Sources and production of laccase

Laccases have been identified in fungi [13, 95], higher plants [96, 97], bacteria [49, 98], and insects [99, 100]. The laccases extracted from the various sources have shown different biochemical properties dependent on the source due to the different roles they play in these organisms [101]. In plants laccases aid in lignin polymer formation, in fungi morphogenesis, stress defence, and lignin degradation, while in insects it participates in stress defence, the biosynthesis of brown melanin like pigment, and protection from UV light and hydrogen peroxide [102]. The laccase from plants and fungi is mainly extracellular while that from bacteria is mostly intracellularly localized [103]. Laccases from fungi have lower optimum pH, ranging from 3.6 – 5.2, than plant laccases that have optimum pH in the range 6.8–7.4. The low optimum pH of fungal laccases is due to their adaptability to grow under acidic conditions, while the plant laccase being intracellular have their pH optima nearer to the physiological range. As a result, the functions of laccases obtained from these sources differ in that fungal laccases mainly degrade toxic phenolic compounds while plant laccases are mainly involved in synthetic process such as lignin formation [5].

Different fungi have been used for the production of laccase including *Agaricus* sp. [93], *Trametes* sp. [104, 105], *Cerrena unicolor* [106, 107], *Dichomitus squalens* [108], *Aspergillus oryzae* [109, 110], *Pleurotus ostreatus* [111, 112], *Coriolus hirsitus* [113], *Coriolopsis* sp. [114, 115], *Rigidoporus lignosus* [116], *Neurospa crassa* [31], *Pleurotus florida* [117], *Cyathus bulleri* [118], *Ganoderma lucidum* [119], *Funalia trogii* [120], *Paraconiothyrium variabile* [121] and *Trichoderma harzianum* [122]. Laccase producing fungi are mainly from three genera, that is, ascomycetes [14], deuteromycetes [123] and basidiomycetes [13]. The basidiomycetes class includes various wood and litter decomposing and soil inhabiting fungi that have been broadly used to produce laccases with variable induction mechanisms, degrees of polymorphism, and physiochemical and kinetic properties due to their efficiency in lignin degradation [108, 112].

Recently, some bacteria have been used as sources of laccase e.g. *B. subtillis* [124, 125], γ-*Proteobacterium* JB commonly found in industrial waste water [126], *Streptomyces coelicolor* [127], *Thermus thermophilus* [27], *Weissella viridescens* [128], *Shewanella putrefaciens* [103], *Alcaligenes faecalis* [129], *Sphingobacterium* ksn-11 [130], *Brevibacterium halotolerans* N11 [131], and *E. coli* [27]. Laccases obtained from bacteria have unique characteristics compared to the fungal and higher plant laccases which gives them greater advantages in terms of industrial applications. For example, the laccase obtained from *T. thermophilus* demonstrated a very high optimal reaction temperature of 92 °C and a half-life of thermal inactivation at 80 °C of over 14 h, making it the most thermophilic laccase reported thus far [27]. Endospore laccases from bacteria serve microorganisms to survive harsh conditions hence the enzyme is expected to withstand harsh conditions such as high temperature, extreme pH, and presence of ionic salts which is beneficial for industrial applications [130, 132].

In plants, laccases have been obtained from maize seeds [133], *Rhus vernicifera* [25], papaya (*Carica papaya*) leaves [67], Sycamore Maple (*Acer pseudoplatanus*) [96], and vegetables such as cabbages, turnip, potatoes, pears, and apples [5, 30]. For example, Bailey *et al.* [133], extracted laccase from maize seeds. A portion of the extractable laccases was in an inactive form and was activated by treatment with copper and chloride.

Laccase is obtained through fermentation of laccase producing organisms. However, the enzyme is usually produced in small amounts and its productivity is increased by introduction of inducers or altering cultivation conditions [134]. The two most critical components of nutritional medium are nitrogen and carbon [42]. Presence or absence of inducers, induction time, nature and

composition of culture medium, carbon to nitrogen ratio, type of culture conditions (pH, temperature, aeration), and genetic manipulation determine the amount and type of laccase to be obtained [135, 136]. Inducers such as 2,5-xylidine [135], benzyl alcohol [49], or xylan [93], their chemical nature, quantity and time of their addition influence laccase production to a great extent. For example, when Osiadacz *et al.* [18], added 10 μM pyrogallol as inducer on the fourth day to the submerged culture, there was a three-fold increase in laccase production from *Trametes versicolor*. Therefore, medium optimization is an important factor to ensure maximum enzyme yield with minimal production costs.

Industrial and agricultural byproducts such as corn cobs [137], olive oil mill waste [138], oak dust [139], banana peel [140], pistachio shell [141], tea residues [142], spent coffee, rice straw, and wheat bran [143] have been used as excellent substrates in the fermentation process due to their complex composition of carbon, nitrogen, and mineral supplies suitable for the growth of micro-organisms. They also possess natural polymers including cellulose, lignin, pectin, and starch which provide suitable substrates during the fermentation process [144, 145, 146]. In fact, fermentation of some white-rot fungi in the presence of lignocellulosic residues significantly stimulated laccase secretion without any supplementation with inducers [147]. For example, laccase was produced by cultivation of *T. versicolor* on barley husks and egg shells in solid state conditions without the need for additional carbon and nitrogen sources or mineral compounds [66]. The eggshells acted as supports for microorganism growth and improved the porosity of nutrient media which enabled more oxygen to diffuse deeper inside the reaction mixture. More so, the egg shells were a source of calcium which acted as a laccase inducer. Production of laccase from various sources has been extensively reviewed elsewhere [148].

5. Applications of laccase

Laccases have attracted attention as biocatalysts in a wide range of biotechnological applications due to their non-specificity and their use of molecular oxygen as an electron acceptor [32, 149]. They have been used as biocatalysts in wastewater treatment of textile, paper and pulp, petrochemical effluents, food industries, medical diagnosis and manufacturing, lignin modification, and as a biore-mediation agent for herbicides and pesticides [150, 151]. Laccases are one of the most promising enzymes for decontamination of phenol polluted systems and biotechnological applications, they have been explored in the removal of toxic compounds from aquatic and terrestrial systems [87], production treatment of beverages [152], region-specific bio-transformations [153], aerobic oxidation of benzyl alcohols [154], analytical tools and biosensors [49], and commercial polymerization of lignosulfates for further applications as surfactants, dispersants and plasticizers in the cement and concrete industry [26]. In this section the various applications of laccase enzyme in waste water treatment, biosensors, paper and pulp industry, food industry, biofuel cells, and pharmaceutical industry are represented in Figure 3 and further discussed.

5.1. Waste water treatment

Enzymatic bioconversion technologies have attracted growing interests in the field of wastewater treatment due to their ability to eliminate toxic compounds at mild conditions with less biodegradation by-products [155]. The use of enzymes for wastewater

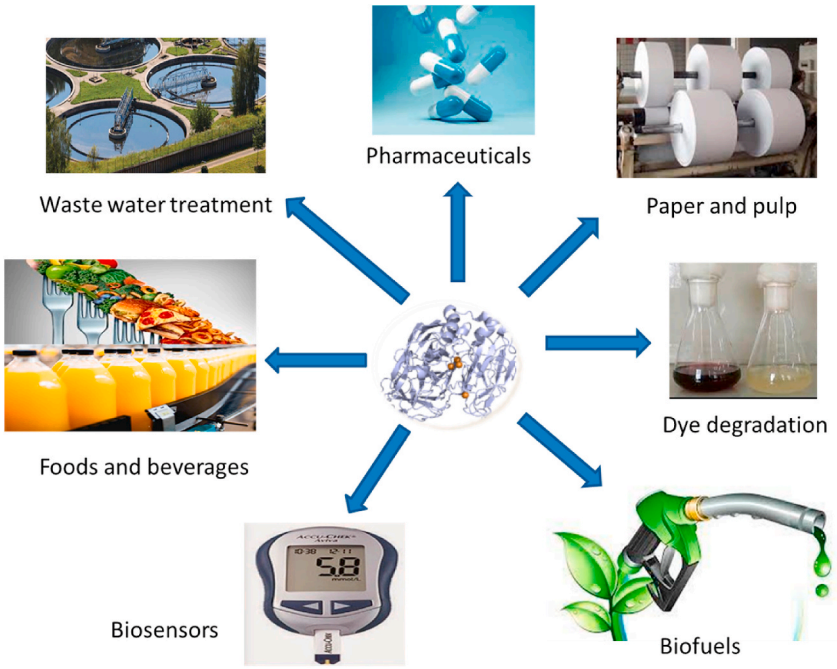

Figure 3. Applications of laccase enzyme.

treatment is advantageous due to the substrate specificity of enzymes, reduced susceptibility of the biocatalyst to shock-loading effects, high rates of reaction, efficient use of oxidants, and effective treatment at low substrate concentrations [156]. The removal of phenolic compounds from industrial effluents and the environment is of great importance as these compounds are highly toxic to aquatic organisms and account for the biggest percentage of organic pollutants [157, 158]. Phenolic compounds form a significant percentage of industrial effluents due to their wide use in many industrial processes such as manufacture of plastics and resins, wood preservation, petroleum refining, paper, dyes, pesticides, textiles, drugs, and anti-oxidants [159, 160, 161]. Majority of these compounds are not removed from the liquid portion by waste water treatment plants and they are transferred into the sludge. More so, they are not degraded by the active sludge in treatment plants hence end up in the environment via waste water effluents and in bio-solids through agricultural applications [162, 163]. These compounds have been related to health problems and environmental pollution due to their inherent toxicity [164]. Consequently, the need to degrade or transform these compounds into degradable or non-toxic ones using enzymes is of great interest [165].

The ability of laccase to degrade a variety of phenolic compounds without harsh side effects has attracted attention in detoxification of polluted waste waters [166, 167]. The enzyme doesn't require exotic co-substrates, uses readily available oxygen as electron acceptor, and the free radicals formed by laccase oxidation bypass the steps involved in formation of carcinogenic amines [168]. Mostly insoluble products are formed when phenol derivatives are oxidized by laccase and can easily be removed by filtration or sedimentation [157, 169]. Laccase has proved to oxidize recalcitrant chemicals from the environment which are difficult to degrade to more bioavailable or harmless and stable water insoluble polymers [29, 83]. For example, High-performance liquid chromatography (HPLC) indicated that the main product of anthracene oxidation by laccase was 9,10-anthraquinone which is less toxic than its precursor [170]. As a result, laccase has been widely used in decontamination and restoration of hazardous chemical-contaminated waters [83, 114]. The mechanisms of free and immobilized laccase in detoxification of phenolic compounds have been reviewed elsewhere [171].

Laccase has been utilized in decolourisation of effluents from olive oil mills [116, 172], molasses wastewaters and baker's yeast effluents [173], and textile, paper and pulp effluents [174]. Degradation of pharmaceutically active compounds such as Diclofenac [175, 176], Tetracycline [177], Sulfamethoxazole [178, 179], Chlortetracycline [180], and carbamazepine [181, 182] from the environment using laccase has been reported. A number of researchers have reported successful laccase mediated degradation of endocrine disrupting chemicals such as bisphenols [114, 183, 184, 185], alkylphenols [186], Polycyclic aromatic hydrocarbons (PAHs) [187, 188, 189], benzidine-based dyes [190], and synthetic estrogenic compounds [156]. Herbicides, pesticides, and insecticides such as 2,4-dichlorophenol [191], Chlorpyrifos [192, 193], lindane [23], pirimicarb [194], triclosan [195], pentachlorophenol [196], as well as other phenolic compounds like naphthalene [17], phenol, p-chlorophenol, and catechol [197, 198, 199] have also been successfully degraded by laccase (Table S1). The application of laccase in waste water treatment and detection of harmful

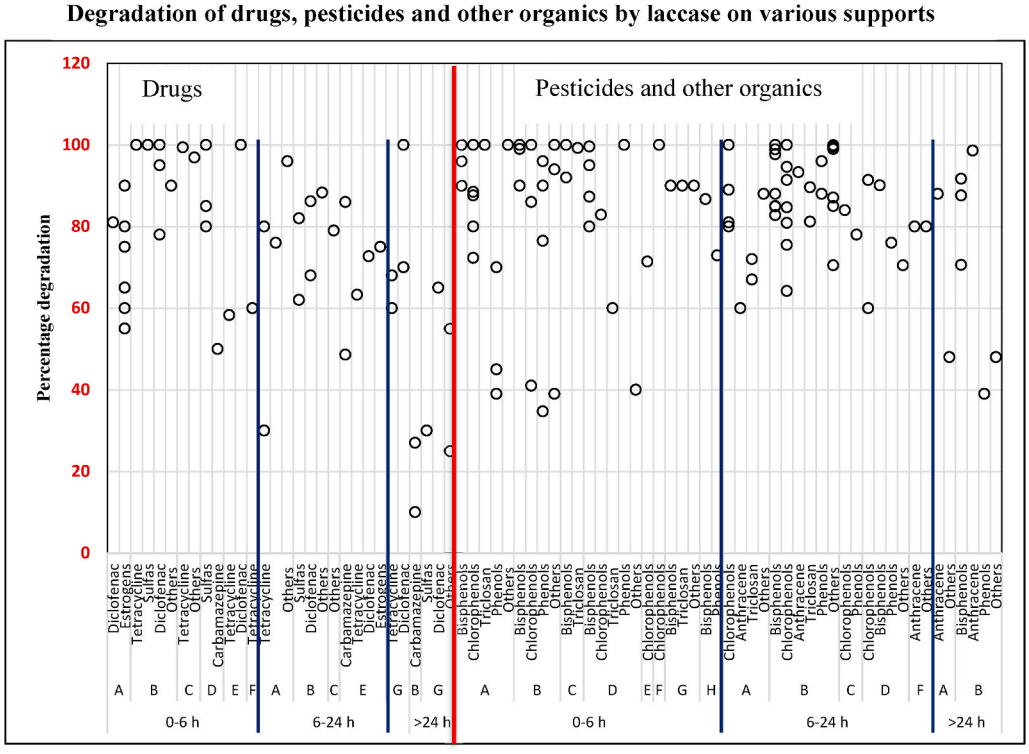

Figure 4. A graph showing the percentage degradation of various drugs, pesticides, herbicides and other organic pollutants that have been degraded using laccase enzyme immobilized on various supports with different times of exposure, that is, 0–6 h, 6–24 h and over 24 h.

pollutants in the environment has been reviewed elsewhere [200, 201, 202, 203, 204].

Figure 4 shows the different drugs, pesticides, and organic pollutants that have been studied for possible degradation by laccase immobilized on various supports. Different drugs such as tetracycline [177, 205, 206], diclofenac [176, 207], estrogens including estrone, 17β-estradiol, 17α-ethinylestradiol [208, 209], sulfa drugs including sulfamethoxazide, sulfadiazine, sulfamethazine and sulfathiazole [80, 210, 211], and carbamazepine [181, 182, 212]. Among other drugs explored are paracetamol [163, 213], hydroquinone [214] and antibiotics such as ampicillin, erythromycin [177], amoxicillin and ciproflaxin [215]. Laccase immobilized on nanoparticles (NPs), nanocomposites (NCs) and metal organic frameworks (MOFs) show the highest degradation efficiencies of over 80% even with short incubation time (0–6 h) as compared to other supports like polymers, silica, and clay. This could be due the high surface area to volume ratios provided by the NPs and NCs which enhances the catalysis of the enzyme [216]. Also immobilization of the enzyme on MOFs is usually by entrapment based on enzyme-MOF affinity which has minimal enzyme denaturation and high enzyme loading hence facilitating high enzyme activity [217].

However, the degradation rate of carbamazepine is low (less than 60%) regardless of reaction time or immobilization support used. Degradation in presence of mediators in the drug solution or co-immobilized on the support can be explored to improve the degradability of this drug since the degradation has been studied exclusively at room temperature in stirred reactors systems [155, 181, 218].

For the case of pesticides and other organic pollutants, bisphenols (A,B and F) [183, 219, 220], chlorophenols [22, 221, 222], triclosan [64, 147, 195], anthracene [77, 223, 224], and other phenolic compounds such as naphthalene [17], catechol [225], phenol [226], and aminophenols [227]. Other pesticides explored include pyrometryn, atrazine [228], indole [223] and chlorpyrifos [192]. As compared to drugs, laccase shows generally higher degradation efficiency for pesticides and other organics regardless of immobilization support or reaction time. This could be due to the phenolic structure of these compounds that provide wonderful substrates for the catalytic activity of the enzyme [10, 11]. It is evident that increasing reaction time from 0-6 h to 6–24 h increases the overall performance of the enzyme in degrading pesticides from below 60% to above 60% with majority above 80% efficiency in degradation. Increasing reaction time above 24 h has limited effect on the performance of the biocatalyst and other options such as use of mediators should be explored.

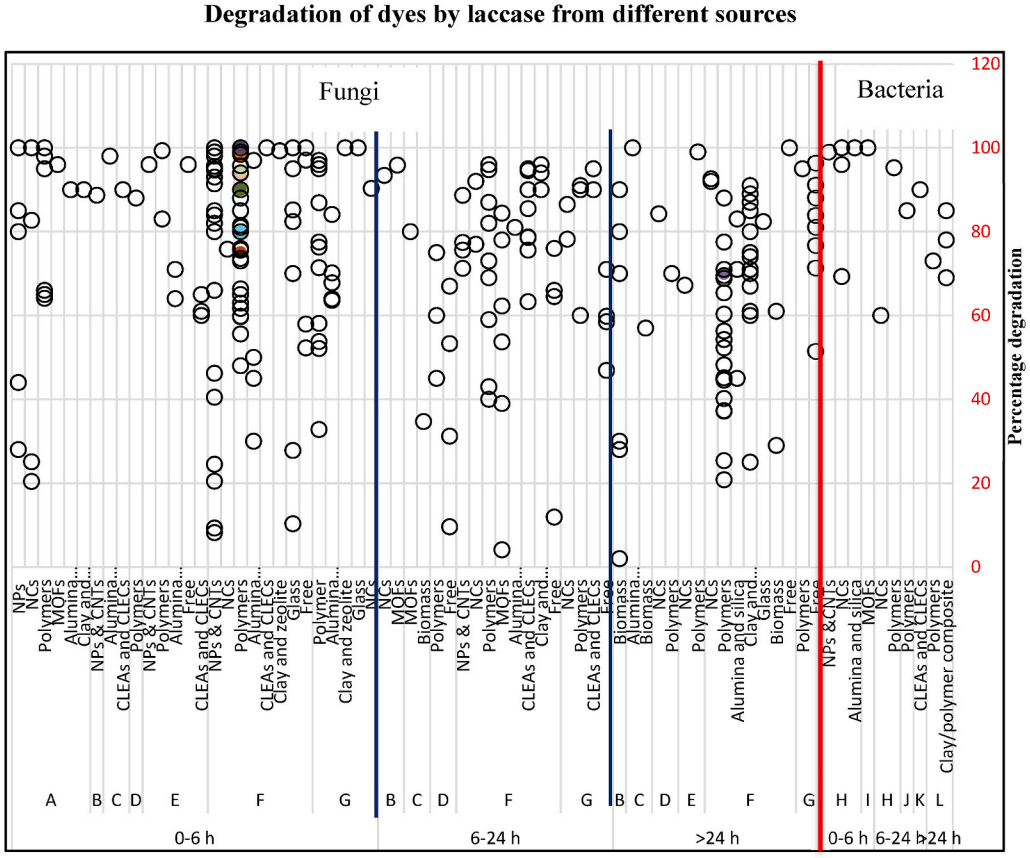

Figure 5. A graph showing laccase immobilized on various supports and its efficiency in degrading synthetic dyes, textile effluents, olive mill waste water and paper industry effluents at different hours of exposure, that is, 0–6 h, 6–24 h and over 24 h.

5.2. Dye degradation

Synthetic dyes have gained popularity in the textile, paper, cosmetics, leather dyeing, colour photography, pharmaceutical, and food industries due to their low cost, ease of synthesis, and colour variety [111, 162]. They are classified according to chemical structure of the chromophore (molecule responsible for their colour) groups as azo, anthraquinone, heterocyclic, triphenylmethane, xanthene, acridine, or phthalocyanine [229]. Azo dyes constitute of the largest percentage accounting for 60–70% of all the dyes in the textile industry [230, 231] followed by the anthraquinones [232]. Since the dyes are designed to meet various colouring requirements, they have very stable chemical structures that are difficult to degrade hence causing serious environmental pollution [198, 233]. They are obstinate to microbial degradation and conventional treatment methods, affecting water transparency and gas solubility leading to reduced dissolved oxygen and may be transformed to carcinogenic compounds under anaerobic conditions [234, 235, 236].

The textile and dye industry is the major source of water pollution with its effluents containing 10–30% of the initial dyestuffs that are difficult to treat due to their non-biodegradable nature and complex molecular structures [168, 237]. The most crucial step in dye degradation by laccases is the cleavage of their chromophores which renders the dye fragments more susceptible to biodegradation by other less specialized biocatalysts [111, 234]. The chemical structure and type of substitute group such as azo, nitro, and sulfo groups of the reactive dye molecule determine the dye decolourisation rate [235, 237]. Biological treatment processes of effluent dye are inefficient because the dyes are highly resistant to biological oxidation and they poorly adsorb on activated sludge [66]. Decolourisation using physical or chemical methods such as adsorption, precipitation, chemical degradation, and photo-degradation has financial and methodological disadvantages, is time-consuming, and mostly ineffective [238]. With the stringent regulations and restrictions established for effluent discharge into the ecosystem, a number of studies have focused on microorganisms, which are able to decolourise and biodegrade these dyes [239, 240, 241, 242]. Enzymatic treatment of dye effluents has gained attention because of the mild reaction conditions involved without generating any secondary pollution like toxic sludge [243, 244].

Laccases have emerged as an attractive enzyme for dye degradation because of their ability to mediate coupling reactions which form a basis for dye removal [43]. Most of the commercially relevant dyes have similar structures to lignin substructures which likely enables the enzyme to act on the compounds chromophore hence decolourising a wide range of synthetic and organic dyes even at environmentally relevant concentrations [245, 246]. Laccases have decolourised a wide spectrum of dyes (Table S1) including Malachite green (MG) [247, 248], Congo red [249], Crystal violet dye [244], Direct blue [250], Procion red MX-5B [231], Reactive red [198, 251], reactive blue [252], Phenol red [253], Acid blue [241], Acid orange [121], Remazol brilliant blue R [232], Reactive green, Reactive brown, Cibacron blue [254], bromaminic acid [255], Direct green [256], Bismark brown R, and Lanaset grey G [115]. The application of laccase enzyme in degradation dyes has been extensively reviewed elsewhere [51, 257, 258, 259].

Although bacterial laccase has not been widely explored, they demonstrate overall high degradation efficiencies of over 60% regardless of the immobilization support and reaction time (Figure 5). Amongst the fungi, *Aspergillus* sp., *M. thermophilia,* and *Cerrena* sp. laccases show higher degradation on all the supports except for biomass immobilized that give less than 40% efficiency. The most explored fungus for laccase production is *Trametes* sp. and their laccases provide desirable degradation rates overall for dyes and industrial effluents in the free and immobilized state.

Even though laccase-NPs/NCs biocatalysts showed high degradation rates for drugs and pesticides at in short incubation times, a longer time (6–24 h) is needed for them to achieve higher degradation efficiencies in dyes and industrial effluents (Figure 5). Polymers have been the most extensively used supports for laccase immobilization and subsequently its application in waste water treatment and their biocatalysts show desirable degradation efficiencies. As observed earlier, increasing reaction or contact time of polymer-laccase biocatalyst with waste water beyond 24 h has no impact on the improvement of the performance of the biocatalyst. In fact, their degradation capacities decrease and degradation efficiencies of below 40% have been recorded for over 24 h incubation. Other supports such as clay, silica, alumina, zeolite and glass provide desirable platforms for the catalytic performance of laccase. Addition of mediators and degradation at laccase optimum pH and temperature can be utilized to improve and facilitate complete degradation of dyes and industrial effluents by laccase. The different supports used to immobilize laccase from various sources and their efficiency in degradation of dyes, paper and textile effluents and olive mill waste water have been showed in Figure 5.

5.3. Paper and pulp industry

Laccase is used in the pulp and paper industry for lignin degradation, deinking, pitch control, effluent detoxification, bio-pulping, and grafting on fibres to improve the physical, chemical, and mechanical properties of paper [260]. In order to obtain high quality paper, structurally embedded lignin responsible for the dark colour should be removed after cooking. This is traditionally done by bleaching with chlorine based chemical agents which results in production of various chloro-organic derivatives that are toxic once released in the environment [261]. Laccase offers a green alternative in bioleaching of pulp to conventional and environment non-friendly chlorine and chlorine based bleaching and has no reductive effect on the final yield of pulp as compared to other enzymes (xylanases and mannanases) [262]. Use of laccase in the pulp industry has showed improved pulp properties, reasonable decrease in kappa number (residual lignin content), increased paper brightness, and reduced chlorine consumption [102]. Laccase has also been used in lignin removal from biomass such as kraft pulp [234] and olive pomace [226] to help in its recycling and enhance the efficiency of cellulose extraction and hydrolysis. The applications of laccase in the paper and pulp industry have been extensively reviewed elsewhere [263, 264, 265].

5.4. Food and pharmaceutical industries

Laccase has found application in food industry as a food additive for stabilization of beverages, wines and beers [266, 267, 268], clarification of fruit juices [269, 270, 271], baking [272, 273, 274, 275], sugar beet pectin gelation [276], and improvement of food sensory parameters [277]. It is also used in the detection of phenolic compounds in beverages, wines and beers [278], as well as bioremediation of food industry waste water. The application of laccase in the food industry has been extensively reviewed elsewhere [277, 279, 280].

Laccase has been used to mediate coupling reactions for synthesis and modification of pharmaceutical products and food supplements such as Trans-Resveratrol [281], degradation of pharmaceuticals from waste water (Figure 4) [218, 282] and catalytic synthesis of anti-cancer drugs and antimicrobial applications [283]. For example, Sampaio *et al.* [284], used entrapped *M. thermophila* laccase in bacterial nanocellulose membrane (from *Gluconacetobacter xylinum*) in wound dressing application. The bioconjugate demonstrated cytotoxicity acceptable for wound dressing applications and 92% and 28% antimicrobial activity against gram positive and gram negative bacteria respectively. The antimicrobial activity of laccase against gram positive and gram negative bacteria was attributed to the electrochemical mode of action to penetrate cell wall of the microorganisms, thereby causing leakage of essential metabolites and physically disrupting other microbial key cell functions.

5.5. Biofuel cells

The fascinating character of direct four electron reduction of oxygen to water by laccase at high electrode potentials has promoted its application in the cathode compartment of biofuel cells [285, 286, 287]. Compared to metallic catalysts, laccase has unique advantages such as catalytic efficiency at high redox potentials, clean oxygen reduction without the formation of hydrogen peroxide intermediate, and relatively low cost [65, 288]. The effective immobilization of laccase and its mediator on electrodes as required is the key step in construction of biofuel cells [289]. Efficient electron transfer between the active site of laccase and the electrode and efficient supply of laccase with oxygen are the prerequisites for high performance of the biofuel cell [285].

Biofuel cells using enzymes as biocatalysts are emerging as the new non-polluting and renewable electricity sources that respect the standards of "green energy'' [124]. Enzymatic biofuel cells take advantage of selectivity of enzymes to oxidize specific substrate and reduce oxygen in order to obtain power output from physiological fluids [290, 291]. Due to the selectivity of enzymes, separation of electrodes in an enzymatic biofuel cell is not required hence the anode and cathode can all be immersed in one membrane containing the fuel and oxidant. This allows for fabrication of miniaturized fuel cells as small as the micrometre scale [292]. For example, Barrière *et al.* [293], used an osmium-based redox polymer for laccase-mediated reduction of oxygen coupled to glucose oxidase-mediated oxidation of glucose to form a membrane-less biofuel cell. At the biocathode, laccase is wired on to an electrode surface to achieve oxygen reduction through either mediated electron transfer (MET) or direct electron transfer (DET) [290]. MET involves integration of redox mediators that shuttle electrons between the active sites of the enzyme and the electrode while DET allows electron transfer between laccase and electrode via fast tunnelling in absence of a mediator [45, 294].

In MET, the mediator (e.g. Dopamine [295] and ABTS [291]) is either free in solution or immobilized with the enzyme on the electrode [296]. For example, laccase was immobilized on three-dimensional graphene networks (3D-GNs) functionalized with dopamine and 3,4,9,10-perylene tetra carboxylic acid (PTCA) which acted as mediator and spacer respectively on the GCE. The modified electrode was used as a cathode in a glucose/O_2 biofuel cell with a maximum power density of 112 μWcm-2 and a short circuit current of 0.96 mAcm-2 [289]. Barrière *et al.* [297], fabricated a biofuel cell consisting of a glucose-oxidase based anode and laccase based cathode. The enzymes were immobilized on graphite electrodes using osmium-based redox polymers as mediators and the biofuel cell gave maximum power density of 16 μW/cm^2 at a cell voltage of 0.25 V. The redox mediators should have excellent reversible electron transfer properties, potentials close to the redox potential of the enzyme active site, and ensure sufficient driving force to optimize bioelectrocatalytic activity [285]. Even though utilization of redox mediators results in higher current densities, MET-based systems often experience mediator leaching, oxygen sensitivity of the mediators, and loss of open-circuit potential [45, 298]. As a result DET-based systems are preferred for high power biofuel cells that can be used in portable power applications [298].

In DET-based cell, the reduction of oxygen relies on the close proximity (within 1.5 nm) of the enzyme active site and the electrode surface [45, 299]. Electron transfer occurs during catalytic transformation of the substrate and its kinetics is determined by the orientation of the enzyme on the electrode surface, potential difference, and distance between the enzyme active site and the electrode surface [298, 300]. DET is optimized when active site of the enzyme molecules are most exposed to the electrode surface and can be improved by modifying the electrode surface with conductive nano-elements like carbon nanotubes and metal and metal oxide nanoparticles [301]. Electrode surfaces modified with nanoparticles such as nano-Gold (nAu) provide a microenvironment similar to that of redox-enzymes in native systems and allow freedom in enzyme orientation since electron transfer proceeds through the conducting tunnels of the nanoparticles [302]. DET is preferred because the biofuel cell voltage is maximized since enzymatic catalysis is performed at apparent redox potentials of the enzymes at the cathode and anode. Also the absence of redox mediators simplifies the fabrication process of the cell, increases cell stability, and minimizes toxicity issues that could arise from the leaching of mediators [285, 303]. Gellet *et al.* [298], used *Rhus vernificera* laccase to develop an air-breathing biocathode that employed DET for application in a proton exchange membrane hydrogen/air fuel cell and a direct methanol fuel cell (DMFC) with an anion exchange membrane. The biocatalyst provided high operational current density of 50 mA/cm^2, maximum power density of 8.5 mW/cm^2, and a lifetime of 290 h in a 40% methanol DMFC. A stable current for 350 discontinuous h when operated for 8 h daily was achieved in the hydrogen/air fuel cell.

5.6. Biosensors

The release of phenolic compounds by a large number of industries necessitates their detection and quantification in the environment. Since these compounds are toxic and persistent in the environment, their quantification is important when evaluating the total toxicity of an environmental sample [304, 305]. Phenolic compounds are also present in fruits and vegetables and their products such as juices and wines. The polyphenol content in juices and wines affects their quality in terms of colour, flavour, stability, and aging behaviour hence the need for their quantification [306]. Most analytical methods used to qualitatively and quantitatively determine polyphenols are expensive, time consuming, and require several operation steps, costly reagents, and separation steps with large amounts of environmentally unfriendly effluents [304, 307].

Biosensors modified with laccase have been developed for detection of laccase electron donors (phenolic compounds) [116, 308] and monitoring oxygen and laccase inhibitors [24]. The construction of laccase-based biosensors is relatively simple as it doesn't require hydrogen peroxide as a co-substrate or any other co-factor for its catalytic activity [164, 309]. The biosensors are based on the principle of reduction of oxygen by laccase to water hence monitor the consumption of oxygen during oxidation of the analyte [43, 308]. The response time is dependent on the enzymatic reaction kinetics, the diffusion and solubility of the substrates at the electrode, and the electrochemical reduction of the oxidized mediator at the electrode surface [310].

Laccase-based biosensors have been fabricated for detection of various compounds including Catechol and its derivatives [164, 305, 311], guaiacol [302], hydroquinone [312], adrenaline [296], Methyldopa [313], Rutin (flavonoid) in pharmaceutical formulations [314], methomyl (insetcide) in vegetable extracts [109], azo-dye tartrazine [15], and industrial kraft lignin [315] as demonstrated in Table 1. For example, Jarosz-Wilkotazka *et al.* [308], immobilized *C. unicolor* laccase on the surface of graphite electrode by physical adsorption and the electrode was inserted into a flow-injection system to serve as a biosensor for detection of polyphenols including flavonoids. The biosensor was more sensitive to bulky highly polymerized polyphenolic structures. The laccase biosensors demonstrate great stability in reusability and storage over long periods. For example, laccase/Nafion biosensor demonstrated 92% activity after 98 days storage [1] and a carbon/laccase biosensor for catechol retained 100% after 90 days storage at room temperature and after 25 assays [316]. Chawla *et al.* [317], for detection of guaiacol, reported retained activity of 85% on a laccase/NiNPs/MWCNTs/PANI/Au biosensor in a space of 4 months and 80% after 300 assays over 7 months for laccase/-CuNPs/CS/MWCNTs/PANI/Au biosensor [318]. Similarly, Cadorin-Fernandes *et al.* [109], reported 100% after 450 assays over 2 months for laccase/sol-gel/carbon ceramic electrode for detection of methomyl.

In order to improve the biosensor characteristics such as minimize laccase leakage and improve laccase-electrode electron movement, a variety of materials have been co-immobilized with laccase on the electrode either by crosslinking or physical adsorption [164]. For example, Rawal *et al.* [353], covalently immobilized *Ganoderma sp*. laccase on a silver nanoparticles (AgNPs)/carboxylated multiwalled carbon nanotubes (cMWCNT)/polyaniline (PANI) layer modified gold (Au) electrode and used it as biosensor for detection of phenolic content in tea, alcoholic beverages, and pharmaceutical formulations. The biosensor gave a linear range, response time, and detection limit of 0.1–500 μM, 6 s, and 0.1 μM, respectively and retained 80% of its activity after 200 reuses for a period of over 4 months. The PANI provided a protective microenvironment that sheltered the enzyme from leakage and the external environment. Wu *et al.* [354], assembled laccase and ABTS on graphene surface and the bioconjugate was deposited on a GCE for detection of extracellular oxygen released from human erythrocytes. The GC/rGO/Lac biosensor was applied to the detection of dopamine in synthetic urine and plasmatic serum samples, achieving a detection limit of 91.0 nmol L−1 [340]. Jabbari *et al.* [355], covalently immobilized laccase on surface plasmon resonance (SPR) carboxymethyldextran chip to promote its activity towards syringaldazine while at the same time making it inert to ABTS. This showed that the specificity and activity of laccase can be enhanced towards a target metabolite. Applications of laccase in biosensors has been extensively reviewed elsewhere [356, 357, 358].

6. Immobilization of laccase

Despite the many advantages associated with using laccase in industrial settings, limitations such as enzyme instability in varying environmental conditions like pH, ionic strength and temperature, proteolysis, inactivation by inhibitors, and the difficulty to separate the enzyme from the reaction mixture limits its further use in industrial applications [215, 359, 360]. Moreover the high cost of continually discarding the enzyme with treated solutions may be particularly prohibitive [361]. For the potential use of laccase to be increased and its reuse and stability in harsh conditions to be achieved, immobilization is necessary [362, 363]. It is the key to optimizing the operational performance of laccase in industrial processes and especially in non-aqueous media [364]. Immobilized enzymes mimic their natural mode in living cells where they are mostly attached to organelle structures, membranes, and cellular cytoskeleton there by improving their stability [115, 365]. The extent of stabilization depends on the enzyme structure, the immobilization methods, and type of support [366].

Enzyme immobilization limits its freedom to undergo drastic conformational changes which equips it with characteristics such as resistance to thermal and pH changes, improved activity, prolonged half-life, greater variety of bioreactor designs, ease of separation from reaction medium, and reusability for longer periods of time hence reduced cost [367, 368, 369, 370]. For example, When Mukhopadhyay *et al.* [371], immobilized laccase from a psychrophilic bacteria on Cu_2O nanoparticles (CuONPs), the enzyme retained high activity at 4 °C which is lower than its optimum temperature (10 °C). And when lipid functionalized single walled carbon nanotubes (SWNTs) were attached to the CuONPs, it retained activity in very low (4 °C) and high (80 °C) temperatures and great stability under repeated freeze-thaw cycles. Lloret *et al.* [208], encapsulated *M. thermophilia* laccase in a sol-gel matrix based on methyltrimethoxysilane and tetramethoxysilane and the enzyme demonstrated improved pH and thermal stability by 10–30%, increased tolerance to different inactivating agents such as acetone, methanol, zinc chloride, calcium chloride, and sodium nitride by

Table 1
Application of laccase in fabrication of biosensors for detection of organic pollutants.

Laccase source	Immobilization support	Electrode used	Analyte	Detection range (μM)	LOD (μM)	Response time (s)	Storage stability and reusability	Ref
A. oryzae	AuNPs/poly (allylamine hydrochloride) (PAH)	Carbon paste	Dopamine	0.49–23.0	0.26			[319]
-	AuNPs-MoS_2	Glassy carbon electrode (GCE)	Catechol	2 to 2000	2	100	90% activity after 30 days, 97% after 10 cycles and RSD of 1.2% and 0.7% for 10 assays and 5 electrodes	[320]
A. oryzae	PEI-AuNPs	GCE	Catechol Guaiacol Pyrogallol Hydroquinone	0.36–11.00 0.79–17.42 1.74–19.60 2.90–22.00	0.03 0.03 0.14 0.21		80% after 90 days and 150 assays	[302]
C. unicolor	poly (N-isopropylacrylamide) gel	Indium Tin Oxide (ITO)	Oxygen in solutions	Reduction signals of 0.79 V and 0.38 V at T1 and T2 respectively				[321]
T. Versicolor	poly (ethyleneimine) (PEI) microcapsules with p-phenylenediamine (PPD) mediator	GCE	Oxygen in solutions				73% after 6 months of storage	[322]
T. versicolor	Fe_3O_4@*Au nanoparticles*		Catechol	5.0–70.0	2	2400		[199]
	Au/Mxene NPs	GCE	Catechol	0.05–0.15	0.05	10.98	89.6% after 15 days of storage and RSD of 2.21% for 9 assays	[323]
T. versicolor	Bacterial cellulose-AuNPs	GCE	Hydroquinone	0.03–0.1	0.00571		96% after 3 months storage, RSD of 2.65% for 3 electrodes and 3.17% for 3 assays	[324]
R.vernicifera sp.	TiO_2–CuC NFs	GCE	Hydroquinone	1–89.8	3.65	5	107.8% after 1 week and 93.45% after 1 month. RSD of 2.69% and 1% for 3 biosensors and 9 assays respectively	[312]
T. versicolor	MnONPs-graphene nanoplates nanocomposite		Caffeic acid	5–320	1.9		77.1% after 1 week, RSD of 3.97% and 1.84% for 10 biosensors and 3 assays	[325]
	Cu/C NFs	GCE	Catechol	9.95–9760	1.18	5	100% and 95.9% after 4 and 22 days, RSD of 4.35% for 3 assays	[326]
T. versicolor	Nafion	DROPSENS cells screen printed electrodes	Catechol Caffeic acid Chlorogenic acid Gallic acid Rosmarinic acid	1.2–120 3–15 3–15 2–7 3–15	0.43 2.5 2.8 1.55 2.4	100	92% after 98 days, RSD 6.08% for 9 electrodes	[327]
T. versicolor	Mg-MCM-41/PVA	Gold	Catechol	0.94–10.23	0.00531	14	90% after 30 days, RSD 5.2% and 4.6% for 10 electrodes and 30 assays	[328]
T. versicolor	PANI/CMC/cellulose	GCE	Catechol	0.497–2270	0.374	8	98% after 2 weeks, RSD of 2.23% and 3.03% for 5 assays and 5 electrodes	[329]
T. versicolor	PANI	Screen printed electrode (SPE)	Bisphenol A	0.004–4	0.004	100		[330]
	MWCNTs-Ag@ZnO nanocomposites	Carbon SPE	Bisphenol A	0.5–2.99	0.6	25	71.02% after 10 days, RSD of 0.86% for 3 assays	[331]
T. versicolor	Carbon black	Carbon SPE	Catechol	2.5–50	2	0.5	100% after 90 days storage at roomtemperature, 100% after 25 assays	[316]
T. versicolor	MWCNTs	Carbon SPE	Bisphenol A	0.84–12	0.84		87% after 1 month, RSD 5.3% for 3 electrodes	[185]

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Table 1 (*continued*)

Laccase source	Immobilization support	Electrode used	Analyte	Detection range (μM)	LOD (μM)	Response time (s)	Storage stability and reusability	Ref
Coriolus hirsuta	MWCNTs	Carbon SPE	Catechol	0.0–0.102		5	100% after 20 days storage, 77% after 10 cycles	[332]
T. versicolor	CS/silica-magnetic MWCNT	Carbon paste	Catechol	0.1–165	0.034	50	90% after 45 days, RSD 3.01% for 35 assays over 29 days	[333]
T. versicolor	CS-MWCNTs nanocomposite	GCE	Catechol	0.1–50	0.02		92% and 85% after 1 week and 1 month respectively. RSD 3.03% for 20 assays	[334]
Ganoderma lucidium	NiNPs/cMWCNTs/PANI	Gold	Guaiacol	0.1–500	0.05	8	85% after 200 cycles in a space of 120 days	[317]
T. versicolor	MWCNT-COOH/AuNPs-SDBS-PEDOT	GCE	Catechol	0.1–0.5 11.99–94.11	0.11 12.26			[335]
T. versicolor	MWCNTs	Carbon paste	Pirimicarb	0.99–11.5	0.18	1200		[194]
P. ostreatus	SWCNT/C-quantum dots	GCE	17a-ethynylestradiol	0.05–7	0.004		96.8% after 10 cycles	[336]
	Reduced GO (rGO)- Sb_2O_5		Estriol	0.025–1.03	0.011	4	84% and 52% after 1 and 2 months storage. RSD of 2.84% and 4.37% for 10 assays and 5 electrodes	[337]
	Magnetic graphene/PANI (PANI/MG)	GCE	Hydroquinone	0.4–337.2	2.94	5	96.7% after 2 weeks storage. RSD of 4.3% and 1.95 for 3 electrodes and 20 assays	[338]
T. versicolor	Graphene-Cellulose Microfiber	Carbon SPE	Catechol	0.2–209.7	0.085	2	96.8% after 132 h. RSD 2.6% for 5 electrodes	[339]
R. vernicifera	AuNPs-graphene nanoplates	Carbon SPE	Hydroquinone	4–130	1.5		100% after 5 days. RSD of 2% and 3% for 5 assays and electrodes respectively	[278]
T. versicolor			*Mercury	49.9–600	74.8			[55]
T. versicolor	rGO	GCE	Dopamine	0.0–3	0.091		89.7% after 30 days storage, RSD 2.1% for 3 electrodes	[340]
T. versicolor	N-doped carbon hollow spheres (NCHS)/CS composite film	GCE	Calcium lignosulfonate (lignin in kraft effluent)	370–19000	120		95% after 1 week storage	[315]
T. versicolor	polyazetidine prepolymer (PAP)/MWCNTs	SPE	Catechol	0.64–20.73	0.18	10		[341]
T. versicolor	Nafion/MWCNTs	SPE	Catechol	1.36–65.45	0.45	10		[341]
T. versicolor	MWCNTs	SPE	Catechol	2.36–134.73	0.73	10		[341]
Agaricus bisporus	AuNPs	Gold disk electrode (GDE)	Dopamine	0.5–13.0 47.0–430.0	0.029		100% after 1 week, RSD 2.7% and 3.2% for 7 assays and 5 electrodes	[101]
	AuNPs	Carbon SPE	Tartrazine	0.2–14	0.04	120	95.5% and 81.3% after 10 days and 1 month. RSD of 2.37% and 8.54% for 5 assays and electrodes respectively	[15]
	Montmorillonite	GCE	Catechol	1–10	0.89	10	RSD of 3.58% for 10 assays	[342]
T. versicolor	3,3′-Dithiodipropionic acid di (Nsuccinimidyl ester) (DTSP)	Gold	Hydroquinone	3–15	0.89	50	96% and 93% after 50 cycles and 6 weeks storage. RSD of 3.1% for 5 electrodes	[343]
T. versicolor	(3-mercaptopropyl)-trimethoxysilane (MPTS)	Gold	Hydroquinone	0.9–20	0.25	50	99% and 97% after 50 cycles and 6 weeks storage. RSD of 3.0% for 5 electrodes	[343]
T. versicolor		Gold	Hydroquinone	3–15	0.91	50	89% and 87% after 50 cycles and 6 weeks storage. RSD of 2.6% for 5 electrodes	[343]
T. versicolor	PANI	GCE	Catechol	3.2–19.6	2.07			[311]

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Table 1 (*continued*)

Laccase source	Immobilization support	Electrode used	Analyte	Detection range (μM)	LOD (μM)	Response time (s)	Storage stability and reusability	Ref
T. versicolor	MWCNTs-silica spheres	SPE	Dopamine	1.3–85.5	0.42		91% and 86% after 10 and 30 days storage. RSD of 4.7% for 5 assays	[344]
T. versicolor	Titania NPs	Graphite	Catechol	0.75–150	0.75	60	94% and 69% after 7 and 22 days. RSd of 7% for 6 assays	[164]
T. versicolor	polyazetidine prepolymer (PAP)-MWCNTs nanocomposite	SPE	Dopamine Catechol Gallic acid Caffeic acid	0.80–4.13 0.07–2.28 1.07–49.16 0.03–0.79	0.24 0.02 0.32 0.01		50% after 6 days	[310]
T. hirsuta	polyazetidine prepolymer (PAP)-MWCNTs nanocomposite	SPE	Dopamine Catechol Gallic acid Caffeic acid	0.03–6.00 0.01–0.95 0.79–19.9 0.03–0.60	0.008 0.003 0.24 0.01		50% after 6 days	[310]
T. versicolor		GCE	Gallic acid Caffeic acid	0.24–117.6 0.0056–0.56	0.24 0.56	100	50% fater 5 days. RSD of 2.8% for 5 assays	[306]
Ganoderma sp.	CuNPs/CS/MWCNTs/PANI	Gold	Guaiacol	1–500	0.156	4	80% after 300 assays over 7 months. RSD of 2.6% for 6 assays	[318]
T. versicolor	Mesoporous silica	GCE	Catechol	2.0–100	2	120	100% after 50 days storage	[305]
T. versicolor	CS-MWCNTs film	Gold	Rosmarinic acid Caffeic acid Chlorogenic acid Gallic acid	0.91–12.1 0.735–10.5 0.793–6.71 0.79–2.1	0.233 0.151 0.161 0.79	120	90% after 15 cycles. RSD of 5.62% for 10 assays	[345]
Pycnoporus sanguineus	CuTAPc-Fe3O4 NPs	Sensor head with oxygen sensing membrane	Adrenaline	0.2–0.9 0.01–0.09	0.01	30	85% after 1 month storage	[296]
A. oryzae	Sol-gel	Carbon ceramic electrode (CCE)	Methomyl	0.5–12.2	0.2		100% for 450 assays over 2 months. RSD of 5.7% and 4.3% for 10 assays and 5 electrodes	[109]
A. oryzae	CS-tripolyphosphate microspheres	Carbon paste	Rutin	0.6–3.92 5.83–13.1	0.0623 0.712		930 assays over 320 days RSD 3.1% for 9 assays	[314]
A. oryzae	Cellulose acetate	Carbon paste	Methyldopa	34.8–370.3	5.5		90% after 60 days. RSD of 1.5% and 4.3% for 6 assays and 5 electrodes	[313]
T. versicolor	Cu-ordered mesoporous carbon/CS	Gold	Catechol	0.67–15.75	0.67		99% and 95% after 15 and 30 days storage. RSD 2.01% for 6 assays	[307]
Ganoderma sp	Epoxy resin membrane	Platinum	Guaiacol Polyphenols in fruit juices Polyphenols in alcoholic beverages	0.5–50 0.81–1.92 1.9–3.0	0.3	30	60% after 200 assays in 10 months. RSD	[304]
Rigidoporus lignosus	Hydrophilic matrix	GCE	Hydroquinone Catechol Caffeic acid Catechin Quercetin Guaiacol Vanillic acid Pyrogallol Gallic acid (in olive oil mill waste water)	0–500 0–500 0–500 0–100 0–100 0–500 0–500 0–500 0–500		60	100% after 100 working days	[116]
T. versicolor	Methylene blue-mesoporous silica	Gold	Catechol	4–87.98	0.331	4		[346]
Ganoderma sp	AgNPs/MWCNTs/PANI	Gold		0.1–500	0.1	6		[347]

(*continued on next page*)

Table 1 (*continued*)

Laccase source	Immobilization support	Electrode used	Analyte	Detection range (μM)	LOD (μM)	Response time (s)	Storage stability and reusability	Ref
			Phenolic content in tea, alcoholic beverages and pharmaceutical formulations				80% after 200 assays in 4 months. RSD of 2.3% for 5 assays	
γ-Proteobacterium JB	Nitrocellulose membrane		Catechol Catechin L-methyl DOPA	40–90 40–60 30–70			100% after 3 months	
T. versicolor	Zn–Cr–ABTS	GCE	Dissolved oxygen	0.06–4	0.06		RSD 8% for 6 assays	[24]
T. versicolor	Polytetrafluoroethylene membranes	Oxygen electrode	Guaiacol	91.3–400	91.3		40% and 40% after 50 assays and 20 days	[160]
	3-methyl2-benzothiazolinone hydrazone (MBTH) films		Catechol	500–8000	330	600	98% after 2 months storage. RSD 5.3% for 8 electrodes	[348]
Denilite		Platinum	Hydroquinone Homogentisic acid	0.2–35 1–50	0.05 0.3	2	80% after 60 days. RSD 3.1% for 7 assays	[349]
T. versicolor	PVP-CLECs	Gold	Phenols	50–1000		120	40% after 30 cycles	[309]
T. versicolor	PANI	Platinum	Phenol Catechol L-DOPA	0.4–6 0.2–1 2–20	0.4 0.2 2	300		[159]
A. niger	PANI	Platinum	Phenol Catechol L-DOPA	0.4–4 0.4–15 0.4–6	0.4	300		[159]
Agaricus bisporus	PANI	Platinum	Phenol Catechol L-DOPA	1–10 0.4–1.6 1–10	1 0.4 1	300		[159]
T. versicolor		Graphite	Catechol	1–10	0.23		RSD 1% and 11% for 12 assays and 6 electrodes	[87]
R. vernicifera		Platinum	ABTS p-phenylenediamine	0.5–15 0.5–20	0.5 0.05	3	80% after 2 months	[86]
T. hirsute	Nafion	GCE	Hydroquinone	0.1–3	0.035	87	80% after 5 days	[350]
Denilite		Platinum	p-phenylenediamine p-aminophenol (PAP)	0.14–29 0.12–22	0.045 0.04	2	80% after 2 months. RSD for 2.8% and 2.6% for 7 assays for PPD and PAP respectively	[46]
C. unicolor		Graphite	Caffeic acid Prodelphinidin B3 *Epicatechin gallate* Catechin Epicatechin	1–10 1–10 1–10 4–40 2–60	0.56 0.43 0.54 4.36 2.44			[308]
Denilite		GCE	PPD	0.15–30	0.04	2	80% after 2 months	[351]
Botryris cinerea	Gelatin	O2 sensing electrode	Hydroquinone	0–8000			96% after 500 assays	[352]

* used UV-Vis spectroscopy using Caffeic acid as an electron donor.

20–40% and preserved up to 80% activity after 10 cycles. Yinghui *et al.* [372], immobilized laccase on carboxylated crosslinked PVA particles activated by N-hydroxysuccinimide (NHS) in aqueous solution. The bioconjugate showed increased stability in extreme pH conditions where it retained 95% activity when incubated at pH 2 and 13 for 4 and 1 h respectively as compared to the free enzyme that had an optimum pH of 2.4.

The reuse and ease of separation of enzymes after immobilization allow use of enzymes in continuous bioreactor operations which is useful in the production of fine chemicals and bio-treatment of industrial and agricultural wastes [373, 374]. It also enables recycling and reuse of enzymatically treated effluents which drastically decreases water consumption and reduces pollution [111, 375]. The properties of the immobilized enzyme are determine by the properties of the support (such as hydrophobicity, surface charge, density of binding sites, and level of support activation) and the immobilization procedure while the operational efficiency of any immobilization system depends on the amount of enzyme retained by the system [359, 376, 377]. Also, the stability of an immobilized enzyme upon storage and repeated use determine its effectiveness in continuous processes [237]. It is evident that enzymes lose much of their initial activity after immobilization due to changes in diffusion rate, variations in the microenvironment, and non-biospecific interactions especially when binding takes place at the enzyme active site or its vicinity since maximal activity is associated with protein flexibility [241, 379]. For example, Gutiérrez-Sánchez *et al.* [380], observed that covalent immobilization of laccase on aminophenyl-modified carbon nanofibers/carbon nanotubes electrode via amide bonds with the aspartic/glutamic carboxylic acid residue close to the type 1 copper site provided unfavourable laccase orientation for DET.

A suitable support matrix with appropriate structural characteristics and an immobilization strategy that maximizes enzyme-matrix interactions for catalytic efficiency should be considered during immobilization for a stable and active biocatalyst [316, 381]. The enzyme-support interaction is significantly influenced by the properties of the enzyme and the support matrix [382]. There is no universal support matrix that can immobilize all kinds of enzymes and the choice of support and immobilization method depends on the specific features of the enzyme and the application it is devoted to [270, 383, 384]. The ideal support for immobilization is expected to be inert, stable, compatible with laccase, and resistant to microbial attack and mechanical force [313, 383, 385]. The morphology, composition, hydrophobicity, specific surface area and functional surface group of the support material determine the immobilization yield and immobilization efficiency of the biocatalyst which in turn affect its performance [256, 386]. For instance, hydrophilic membranes are preferred as immobilization supports for catalysis under isothermal conditions (because they provide a better microenvironment for enzymes) while hydrophobic membranes are preferred for non-isothermal conditions [190]. Also, the charge and charge density on the support surface are important as they can alter the enzyme activity upon immobilization due to electrostatic interaction [387]. Therefore for a successful immobilization, a suitable carrier and immobilization procedure that maximize the catalytic and non-catalytic needs of a biocatalyst have to be considered [379, 388, 389]. For a successful biocatalyst, enzyme loading, activity and stability, specific activity of the bound enzyme, and storage stability have to be maximized [390].

A good immobilization strategy should maximize the catalytic (stability, selectivity, and space time yield) and non-catalytic needs (separation, control, and down-streaming processing) for a given application [391]. This entails selecting a suitable carrier, considering the nature of the enzyme, and conditions of immobilization [392]. For example, Tastan *et al.* [160], compared immobilization of laccase on polytetrafluoroethylene (PTFE) membranes using entrapment to gelatin and covalent immobilization to the surface using carbodiimide coupling. For covalent immobilization, the functional groups were formed on the PTFE surface by radiofrequency (RF) plasma treatment followed by polymer grafting with polyacrylamide (pAAm) and polyacrylic acid (pAAc) polymers. It was observed that, although the membrane with entrapped enzyme displayed high activity, it had poor mechanical stability, narrow working pH range, and low storage life while the covalently bound membrane showed high stability and reusability. Qiu *et al.* [393], immobilized laccase on nanoporous gold via three approaches: physical adsorption, electrostatic attraction, and covalent coupling. Physical adsorption gave the best results because of the covalent linkage between the nanoscale gold surface and the amino groups of the residue amino acids of laccase. Adhami *et al.* [374], compared covalent immobilization of laccase from three different fungi, *viz. Cerrena unicolor*, *Heterobasidion annosum* and *Trametes versicolor* on DEAE-Granocel 500. *C. unicolor* laccase showed the best binding efficiency to the carrier, good enzyme activity, and thermal and storage stability. On the other hand, *T. versicolor* laccase immobilized on hexagonal mesoporous silica nanoparticles demonstrated higher activity across a broader pH range as compared to when it was immobilized on Kaolinite under the same conditions [166].

Immobilization sometimes leads to alteration of enzyme properties such as catalytic efficiency and optimum pH and temperature. The enzyme kinetic parameters are altered as well facilitated by changes in affinity of substrate to an enzyme due to steric hindrances, partitioning, and diffusion effects of the substrate and decreased protein flexibility [162, 197, 394]. For example, Lante *et al.* [395], adsorbed laccase on a spiral-wound asymmetric polyether-sulphone membrane, the optimum pH changed from 6.3 to 6.6 due to the presence of ionized groups on the support. The change in pH activity of the immobilized enzyme is due to electrostatic interactions especially for charged supports which lead to the unequal partitioning of H^+ and OH^- concentrations between the microenvironment of the immobilized enzyme and the bulk phase [107, 161, 390]. When Abdullah *et al.* [348], immobilized laccase on chitosan-nafion/sol-gel silicate MBTH and used it as a biosensor for catechol, guaniacol, o-cresol and m-cresol, the sensor was responsive only to catechol and the immobilized enzyme had no catalytic effect on the remaining substrates. The non-reactivity of immobilized laccase is attributed to the hydrophobic hybrid Nafion/sol-gel silicate film used for immobilization which restricts the interaction of the enzymes active site with certain phenolic compounds through steric hindrance and change in stability of the phenoxy radical produced.

However, immobilization may not offer any improvement on the enzyme properties. For example, when Abadulla *et al.* [104], immobilized *T. hirsuta* laccase on APTES silanized alumina pellets with GA cross-coupling, immobilization showed insignificant change in enzyme half-life, thermal stability and resistance against inhibitors. In some instances, the immobilization has shown to reduce the enzyme properties. For example, Zille *et al.* [375], immobilized *Thapsia. villosa* laccase on APTES silanized alumina

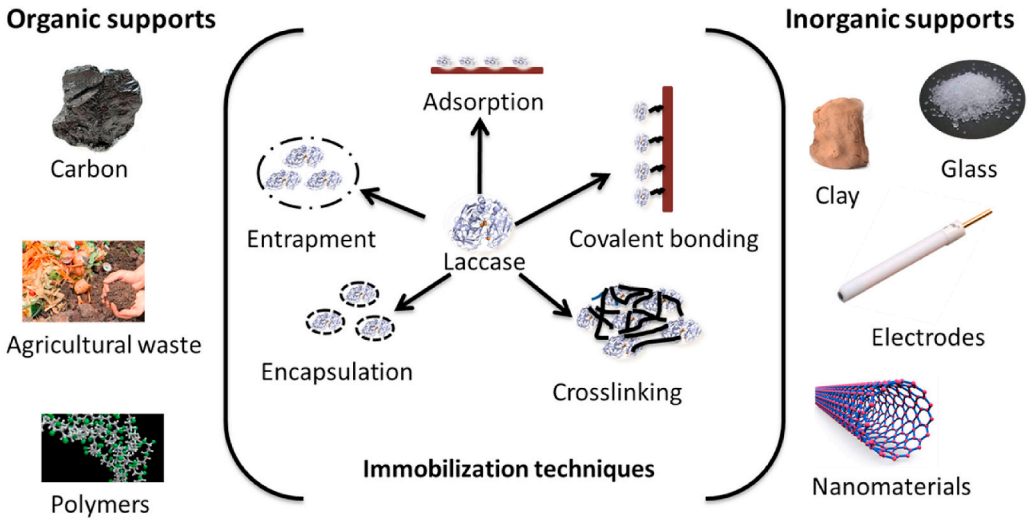

Figure 6. Various techniques and supports used for immobilization of laccase enzyme.

spherical pellets with GA crosslinking and used it for decolourisation of industrial dye effluent. It was observed that decolourisation was predominantly due to physical adsorption of the dye on the immobilization support (79%) and not by the catalytic activity of laccase (4%) and the free enzyme demonstrated more stability than the immobilized enzyme. The high amounts of salts (especially sodium chloride) in dyeing effluents enhanced electrostatic coupling of the anionic dyes and the positively charged proteins thereby forming stable dye/enzyme aggregates for free laccase. However, the restricted enzyme structure limited accessibility for interaction of the enzyme with salts and anionic dyes.

Immobilization conditions such as pH, temperature, concentration of crosslinking agent, concentration of enzyme, and type of solvent also affect the activity of the biocatalyst [359]. Different solvents affect the substrate-enzyme interactions and conformational mobility and structure of catalyst [396]. For example, Addition of organic solvents (20% ethanol, acetone and acetonitrile) in laccase solution during laccase immobilization on SWCNTs registered 600%, 400%, and 350% increase in maximum reductive current, respectively, when the biocatalyst was used in mediatorless electro-reduction of oxygen [45]. It was observed that ethanol promoted laccase-SWCNTs contact leading to favourable enzyme orientation on SWNTs. An increase in enzyme concentration during immobilization offers increased bioconjugate activity up to a maximum beyond which increasing the enzyme concentration leads to decreased bioconjugate activity [397, 398]. This is because an increased enzyme concentration leads to increased enzyme loading up to a point beyond which high enzyme loadings produces intermolecular space hindrance of the immobilized enzyme which screens the active site of the enzyme and restrains the dispersion of the enzyme or damages the active sites [359, 399]. This was demonstrated by Fortes *et al.* [400], where beyond a given enzyme concentration, the enzymatic activity decreased with increases in enzyme concentration. This was attributed to support saturation due to excess enzyme in solution leading to steric hindrance and restrained dispersion of substrate and products. Figure 6 represents the various immobilization techniques and immobilization supports that have been explored for laccase enzyme.

6.1. *Immobilization techniques*

There are five major techniques employed in immobilization of laccase namely, physical adsorption, entrapment, encapsulation, covalent bonding, and crosslinking (Figure 6). The techniques are classified as physical (entrapment, encapsulation and adsorption) and chemical (covalent and crosslinking). These methods vary in cost and ease of preparation, stability, catalytic efficiency, and physical properties of the resultant biocatalyst [401]. The different immobilization techniques with their advantages and disadvantages as well as the type and strength of interaction between enzyme and support for each technique have been summarized in Table 2.

Immobilization with physical techniques involves relatively weak and reversible interactions with the support [365]. They offer less disturbance to the enzyme structure and retain enzyme properties to those in solution [19]. However, the weak interactions between the enzyme and support cause enzyme leaching which results in activity loss and contamination of the surrounding media [212]. And since there is no control over the parking density of the enzyme on the support, the activity of the biocatalyst can be reduced due to overcrowding of the enzyme [365].

6.1.1. *Entrapment*

Entrapment is the physical confinement of an enzyme in the micro-spaces of porous hollow fiber, spun fiber, insoluble gel matrix, and/or a reverse micelle without chemically binding to the support [402]. This approach allows preservation of the enzymes' three-dimensional conformation and optimum operating parameters since there is no interaction between the enzyme active site and support upon immobilization [245]. It also offers practical convenience of simple regeneration by removal of deactivated enzyme

Table 2
Characteristics of the various immobilization techniques.

Immobilization technique	Type of interaction	Strength of interaction	Advantages	Disadvantages
Entrapment	Ionic interactions, hydrophobic interactions, covalent bonds	weak/strong	No enzyme modification, ease of preparation, minimal loss of enzyme activity	Pose diffusion limitations to substrates and products, high enzyme leakage, difficult to implement at industrial level
Encapsulation	Ionic interactions, hydrophobic interactions	Weak	No enzyme modification, protection of the enzyme, minimal loss of enzyme activity, minimal enzyme leakage	Pose diffusion limitations to substrates and products, less concentration of enzyme
Adsorption	Epoxy groups hydrogen bonds, ionic interactions, hydrophobic interactions	Weak	No enzyme modification, simple and inexpensive reusability of the support, minimal diffusion limitations for substrates and products	May pose enzyme leakage, probable activity loss of enzyme, lower efficacy
Covalent binding	covalent bonds	strong	Strong and stable interactions, multipoint attachment, minimal enzyme leakage	Costly to prepare, may pose diffusion limitations for substrates and products, activity loss of enzymes, enzyme once denatured the support and enzyme are discarded
Cross-linking	covalent bonds	strong	No support needed, high strength of interactions	Poor stability, structural modification of enzyme by crosslinker leading to activity loss

reloading with fresh active catalyst [367]. It is the most preferred technique in industries because it is a mild process, easier to operate, and provides minimal structural changes to the enzyme hence retaining the enzyme activity [200, 403]. For instance, When Makas *et al.* [367], entrapped laccase in K-carrageenan based semi-interpenetrating polymer networks, the optimum pH and temperature remained unchanged and the enzyme displayed high stability of 82% activity after 42 days of storage and over 50% activity after 10 reuses.

During this process, a suitable support is required to trap the enzyme molecules or enzymatic preparations within a matrix [404]. The success of this technique is greatly dependent on the size of the pores of the support as pores smaller or equal to the size of the enzyme yield lower loadings and the enzyme simply adsorbs onto the external surface while pore sizes that are much bigger than the enzyme size encourage leaching of the enzyme from the support [405]. In instances where the pore size is relatively larger than the size of the enzyme, adsorption of the enzyme to a porous support is often followed by crosslinking to minimize leaching [406].

6.1.2. Encapsulation

Microencapsulation is a form of entrapment where the enzyme is entrapped in the core of micron-sized spheres made from semipermeable hollow (microcapsule) or solid (microbeads) material [40, 407]. The core of the capsule provides an aqueous environment which support enzymatic activity and the capsule (wall) itself serves as a container through which only the substrates can diffuse [322]. This method ensures the enzyme is fully embed in the supports and prevents interference that can arise from interaction of the enzyme with the external interface [408]. Microencapsulation is especially favourable in electrodes as high amounts of enzyme can be immobilized and the polymer wall properties can be tuned to prevent diffusion of inhibiting molecules in the capsule [322]. This method is economical and allows encapsulation of large volumes of the enzyme but is limited by diffusion limitation of the substrate across the membrane and accumulation or repulsion of the substrate by the membrane [200, 322].

Approaches such as layer by layer (LbL) and self-assembly using polyelectrolytes have been used to encapsulate laccase [322]. The LbL technique involves deposition of enzymes between films in multilayer system through alternating layer by layer deposition of support and enzyme. The advantages with this technique are that a variety of materials can be incorporated into the layers with controlled thickness of the biocatalyst [85, 224]. The enzyme activity is retained without compromising its stability and it can easily be reloaded when necessary [409]. Laccase has been encapsulated in materials like nanocellulose [200], thin silicate films [69], graphene oxide-laccase nanoassemblies [224], self-assembled monolayer (SAM) - 3-mercaptopropionic acid [101], and sol-gels [109, 164].

6.1.3. Adsorption

The adsorption technique involves attachment of laccase on the exterior surface of a support through hydrogen bonding, electrostatic forces, or hydrophobic effects and solely depends on the isoelectric points of the enzyme and support [200, 405]. For electrostatic attraction, the charge on the surface of the carrier should be opposite to the charge of the enzyme and these are determined by the isoelectric point of the enzyme, pH of the buffer, and ionic strength of the medium [383, 393]. Because this technique is associated with low linking energy between enzyme and support, the enzyme is susceptible to leaching during washings and changing pH, temperature, and ionic strength [391]. The active site of the support may be blocked by the support greatly reducing the enzyme activity [410].

To minimize these limitations, metal-chelated and bioaffinity adsorption have gained popularity in immobilizing laccase. Metal chelated adsorption is based on the covalent conjugation of metal ions and His or Cys residues at the surface of the enzyme [411]. This approach is preferred because of its simple operation, stability, reversibility, and oriented enzyme immobilization for spatial accessibility into the substrate [196, 213]. For example, when laccase was immobilized on Cu^{2+} chelated zinc oxide nanoparticles (ZnONPs) and manganese oxide nanoparticles (MnO_2NPs) through metal affinity adsorption, the immobilized laccase demonstrated almost double the catalytic activity towards degradation of alizarin red S dye (95% for lac-ZnONPs and 85% for lac-MnO_2NPs) than the free

laccase [412]. Wang *et al.* [397], achieved 92.5% activity and a retained activity of 86.6% after 10 successive batch oxidation of catechol when *T. versicolor* laccase was immobilized on Cu^{2+} chelated magnetic mesoporous silica nanoparticles via metal affinity adsorption at pH 3.0. Wang *et al.* [413], immobilized laccase on amidoxime polyacrylonitrile nanofibrous membranes (AOPAN) chelated with four metal ions (Fe^{3+}, Cu^{2+}, Ni^{2+}, Cd^{2+}) individually and as a mixture of the metal ions. The membrane chelated with four mixed metal ions showed the best properties followed by the individual metal ions in the order Fe–AOPAN–Lac > Cu–AOPAN–Lac > Ni–AOPAN–Lac > Cd–AOPAN–Lac. The stability of the immobilized enzyme depended on the size and polarizability of the metal ions. The saturation of laccase His structures with metal ions stabilized the enzyme's three-dimensional structure through special covalent coordinate bonds which minimized enzyme distortion and desorption [184, 414].

Bioaffinity based adsorption utilizing the affinity of laccase carbon moieties to the support surface has been explored. For example, Li *et al.* [415], exploited the specific affinity of Concanavalin A (Con A) for glycosyl to immobilize horseradish peroxidase and laccase on the surface of single walled carbon nanotubes (SWNTs) through self-assembly. The SWNTs were incubated with n-dodecyl β-*D maltoside* (DM) to form SWNTs-DM complex which was activated with Con A for enzyme immobilization. DM and the enzymes were bound on Con A step by step via the affinity force to form a sandwich structure hence indirectly immobilizing the enzymes on the SWNTs. This approach allows enzymes to be directly adsorbed from crude homogenates which bypasses the cost of enzyme purification with fewer immobilization residues generated [391].

6.1.4. Covalent

Covalent immobilization methods include enzyme attachment to matrix by covalent bonding, crosslinking between enzyme and matrix and enzyme crosslinking by multifunctional reagents. Covalent immobilization to a support involves interaction between an activated group on the support and a functional group on the enzyme (usually *β*-amino groups of basic residues) [11]. Different functional groups such as carboxyl [379], hydroxyl [390], amino [17, 166], and epoxy groups are added or activated on the support surface which form covalent bond with the enzyme. For example, Salami *et al.* [416], functionalized silica gel with epoxy groups by dispersing it in dry toluene followed by addition of glycidyloxypropyltrimethoxysilane (3-GPTMS) and triethylamine Et3N solutions. The epoxy functionalized silica gel was then added to laccase solution for immobilization. Kashefi *et al.* [417], introduced amino functional groups on graphene oxide (GO) nanosheets by reacting them with APTES in ethanol solution. Glutaraldehyde was then added as a crosslinking agent between the amino residues on laccase and the modified GO.

Protocol for covalent immobilization begins with surface modification or activation, for example carbodiimide activation, silanization of inorganic supports, and derivatization of amino groups to arylamine groups using *p*-nitrobenzoyl chloride or to aldehyde groups using glutaraldehyde [405, 418]. The most common is silanization where the support is coated with organic functional groups using an organofunctional silane reagent [419]. Carbodiimide activation is also used often to form epoxy groups on the surface especially when a carboxyl group is expected to react with the amino groups on the enzyme [19]. Other support activation methods such as plasma polymerization of the support [418] and activation with glutaraldehyde have been reported [378]. The added functional groups on the support surface are then derivatized using coupling or crosslinking agents to react with the amino groups on the enzyme surface to form covalent bonds [404, 419]. For example Kuznetsov *et al.* [420], during the covalent immobilization of laccase on a GCE modified the surface of the electrode with carboxyl groups by treating it with oxygen plasma and used condensing N-cyclohexy-N'-[b-(N-methylmorpholino)ethyl] carbodiimide p-toluene-sulfonate salt (CMC) to activate the carboxyl groups. Laccase immobilization was then achieved by incubating the modified electrode in PBS containing laccase enzyme. Merle *et al.* [68], explored the aspect of molecular recognition for covalent immobilization of laccase on aminopolypyrrole film modified carbon electrodes. The enzyme and aminopropyrrole modified electrode were biotinylated by reaction with excess biotin-amidocarproate NHS ester solution. The biotinylated electrode was immersed in avidin solution followed by immersion in biotinylated laccase solution to form covalent bonds. Labus *et al.* [418], modified the surface cellulosic and polyamide microfiltration membrane by plasma polymerization of allylamine, allyl alcohol, and acrylic acid to introduce amine, hydroxyl, and carboxylic functional groups for covalent immobilization of *C. unicolor* laccase and *A. bisporus* tyrosinase. The hydroxyl groups were then activated with divinyl sulfone, carbodiimide, or glutaraldehyde.

Various coupling agents such as glutaraldehyde, tripolyphosphate [314], Sebacoyl chloride [40], EDC [421], and/or NHS [44, 341, 422] are used to bind the activated support surface and the enzyme. Glutaraldehyde (GA) is one of the most commonly used crosslinking agents during covalent immobilization and also acts as a spacer arm due to its five carbon length [114, 373]. It reacts with the amine groups of enzymes and supports through formation of Schiff's bases and Michael's adducts [423, 424, 425]. One aldehyde group of GA reacts with the amino groups on the surface of the support to form imino groups and the other interacts with the amino groups of the enzyme [388, 399]. GA promotes multi point attachments and enzyme rigidification hence the amine content of the carrier, enzyme type and concentration, pH, and reaction time have to be considered when using it [219]. It has been observed that activity of immobilized enzyme increases with increasing concentration of GA due to the increasing amount of free aldehyde groups on the surface to cause higher loading [359, 388]. At higher glutaraldehyde concentrations, there are extensive interactions of individual enzymes with aldehyde groups which changes the enzyme conformation leading to excessive crosslinking between enzyme and support, aggregation and precipitation of the enzyme, hence drop in activity [348, 399]. Moreover, very high concentrations of GA can induce GA-GA interactions rather than laccase-glutaraldehyde interactions, since GA is a bifunctional reagent [426].

Crosslinking with GA leads to a dimeric or trimeric structure which results in creation of a spacer between the matrix and protein surfaces. The spacer distances the enzyme from the support surface which allows access of the substrate to the enzyme [390]. Patel *et al.* [386], compared covalent immobilization of laccase on silica nanoparticles by functionalization with different functional groups, that is, aldehyde using glutaraldehyde, carbodiimide using 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate, and cyanogen using cyanogen bromide under the same conditions. Aldehyde functionalized support gave

the highest immobilization yield and efficiency (73.2% and 78.4% respectively) followed by carbodiimide (64.5% and 64.2% respectively) and lastly cyanogen (56.45% and 61.4% respectively). However, the high reactivity of GA and its small size enable it to diffuse into the internal structure of the enzyme thereby causing loss in enzyme activity [32]. GA has also been proven to be toxic to aquatic species by reducing reproduction and impeding growth and to human health. When used for crosslinking it may result in leaching from the biocatalysts to the receiving environment where it can cause adverse effects to the aquatic ecosystems [427].

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) is another commonly used crosslinking agent. It is a water-soluble, zero-length heterobifunctional crosslinking reagent that reacts with carboxylates to form highly reactive O-acylisourea intermediates that spontaneously react with the amine groups of the enzyme to form amide bonds [421, 428]. It is a zero-length crosslinker and therefore does not generate extra linkage but dissolves as urea. However, it can promote intramolecular bonding between carboxylates and amines groups of the enzyme instead of intermolecular bonding between enzyme and support. It is also unstable in aqueous solutions as the O-acylisourea is cleaved to an iso-urea before amide coupling which leads to regeneration of the carboxylate [32]. It has also been used as an activating agent in conjunction with other crosslinkers such as chitosan [427] and NHS [65].

Other coupling agents such as NHS [365], glyoxal [32], genipin [429] and epichlorohydrin [366] have been reported. NHS as a crosslinker generates an NHS ester on the enzyme which the reacts with the amine-bearing supports to form covalent bonds [365]. Glyoxal is a dialdehyde often used as a surrogate to GA due to its lower toxicity and reduced steric hindrance [32]. Genipin, a natural crosslinker, reacts with primary amine groups under mild conditions and exhibits biocompatibility with low toxicity [429].

Depending on the enzyme and support properties, different coupling reactions can offer superior immobilization yield and efficiency for laccase. For example, Quan and Shin, 2004 [351] compared four covalent immobilization approaches for laccase on a glassy carbon and platinum electrodes. The first and second approach involved peptide coupling using EDC/NHS of laccase on 1,5-pentanediol modified or bare electrodes. The third approach involved immobilization on hydroxyl functionalized electrode with cyanuric chloride as a coupling agent while the fourth method used APTES silanized electrode with GA coupling. The APTES/GA immobilization technique was the most effective with respect to long term stability and fast response in detection of *p*-phenyldiamine substrate. On the other hand, when Costa *et al.* [430], compared different approaches with combinations of methods involving hydrothermal oxidation with nitric acid, treatment with APTES, GA, EDC, and NHS for laccase immobilization on multi-walled carbon nanotubes (MWNTs), the MWNTs functionalized with HNO_3 and treated with EDC/NHS gave the best thermal stability and immobilization efficiency and yield.

During covalent immobilization, laccase activity may be hindered due to the involvement of amino acid residues near the active site in bonding. To minimize this, small molecules to act as spacer arms are added [39, 80, 85]. Spacers such as β-alanine and polyethyleneimine (PEI) improve the enzyme loading capacity, microenvironment, orientation, and conformational flexibility of the enzyme and in turn improve biocatalytic activity [414, 431]. For instance, Arica *et al.* [373], compared direct immobilization of laccase on poly (GMA/EGDMA) and use of DAH-GA spacer-arm to move the enzyme away from the support and the immobilization with a spacer arm gave a higher activity yield of 83% as compared to no spacer-arm used of 53%. The low activity of the enzyme immobilized directly on the polymeric support was attributed to steric hindrance between the support surface and immobilized enzyme.

Covalent immobilization techniques provide strong stable enzyme attachment with reduced leaching and deactivation rates [362, 432]. For example, Pang *et al.*, compared immobilization of *T. versicolor* laccase on carbon nanotubes by adsorption and covalent immobilization achieved by functionalization of the nanotubes with 1-aminopyrene and GA crosslinking. The biocatalysts were used in oxygen reduction and it was observed that the covalently immobilized enzyme displayed higher electrocatalytic activity and better stability than the adsorbed laccase. As a result this method is preferred for long-lasting, real-scale, and continuous applications [426].

The other advantage with covalent immobilization is that the bonding between enzyme and support can be oriented so as to achieve better biocatalyst activity [80]. For example, Rotková *et al.* [433], explored oriented (through carbohydrate moieties) and non-oriented (through NH_2 groups on the laccase surface) covalent immobilization of laccase on magnetic bead cellulose (size 125–250 μm). For non-oriented immobilization, the magnetic macroporous bead cellulose were oxidized with sodium periodate followed by incubation with laccase in the presence of Sodium cyanoborohydride. In the case of oriented immobilization, the magnetic beads were functionalized with hydrazide functional groups and laccase was oxidized with sodium periodate. The oxidized enzyme was then incubated with the functionalized carrier to form covalent bonds. Although both approaches led to formation of stable hydrazone based bonds, the oriented approach achieved an enzyme activity that was almost three times (0.63 I.U./1 ml of settled carrier) the activity of the non-oriented immobilization approach (0.22 I.U./1 ml of settled carrier).

However, during covalent immobilization, the native structure of the enzyme is usually affected due to the multipoint attachments and the enzyme activity often reduces due to the rigorous treatment with toxic coupling reagents [322, 423, 434]. For example, when Zhu *et al.* [360], immobilized *T. versicolor* laccase on magnetic mesoporous silica spheres by physical adsorption and covalent attachment method (using $–NH_2$ activation and GA crosslinking), the retained enzyme activity for covalent attachment was much lower (56.9%) compared to that of physical adsorption (79.4%). In some instances, the activated groups on the support surface and enzymes may not be compatible leading to very low immobilization yield. For example, Russo *et al.* [435], achieved only 7% immobilization yield when laccase was immobilized via covalent binding between amino, thiol, and carboxyl groups of the enzyme and the epoxy residues of Epoxy-activated acrylic beads, EUPERGIT C250 L. More so, the covalent bonds formed via amino groups of the enzyme reduce its conformational flexibility and may result in higher activation energy for the molecule to reorganize the proper conformation of the binding substrate [373].

6.1.5. Crosslinking of enzymes

The main challenge with carrier bound immobilization is the dilution of catalytic activity as the biggest portion of the biocatalyst is

occupied by the support leading to lower volumetric and space-time yields hence lower catalyst productivity [147, 436]. Moreover high enzyme loading on the support often results in loss of activity and instability towards leaching in aqueous media [437]. Also cross linking reactions between enzyme and support are difficult to control which often leads to structural denaturation of the enzyme molecules [404]. To minimize these effects, self-immobilization techniques where laccase is incubated with crosslinking agents have been explored [81, 436]. And since the molecular weight of the crosslinking agent is negligible in relation that of the enzyme, the biocatalyst can essentially comprise of 100 % active enzyme [147]. Therefore, the immobilized enzyme exhibits operational stability and reusability with efficiencies close to those of the free enzyme [103, 438].

6.1.5.1. Crosslinked enzyme crystals (CLECs). Crosslinked enzyme crystal (CLECs) are prepared by controlled precipitation of enzymes into micro crystals followed by crosslinking using bifunctional reagents to form strong covalent bonds between amino groups of lysine residues within and between the enzyme molecules [32, 438]. The protein in CLECs are stabilized by links in the three-dimensional structure unlike immobilization on a support in which the enzyme is linked to the support by multi-point attachment to a two dimensional solid surface [309]. CLECs display stability in organic solvents, shear stress and temperature and storage conditions [439]. The stability of CLECs in organic solvents is attributed to cross linking which increases the rigidity of the enzyme molecules and reduces the unfolding of the three-dimensional structure of the enzyme by the organic solvents [309, 440]. For example, CLECs showed higher activity in organic solvents (toluene, hexane, cyclohexane, and isooctane), 4-fold thermal stability, and a half-life of 123 min compared to the 24 min of the free enzyme at 60 °C [441]. During the conjugation reaction of catechin with poly (alkylamine), Gogoi *et al.* [442], compared the catalytic activity of free laccase, crosslinked enzyme crystals of laccase, and laccase immobilized on Celite 545 support. CLECs displayed the highest stability in the organic solvents as well as catalytic activity. Their operational stability, controllable particle size, and ease of recycling, coupled with their high catalytic and volumetric productivities make CLECs ideal as industrial biocatalysts [443].

One major drawback of CLECs is high purity enzymes are needed for crystallization which translates to high costs [439, 444]. Also the multi-step enzyme purification is tedious and time consuming and enzyme purification and stabilization should ideally be achieved simultaneously [155]. As a result, crosslinked enzyme aggregates (CLEAs) have gained popularity. The CLEAs are prepared by precipitation of the enzyme from aqueous buffer (using precipitating agents like ammonium sulfate and polyethylene glycol [429, 445] followed by crosslinking of the aggregates with crosslinking agents such as glutaraldehyde [28, 445], genipin [429], chitosan and EDC [427]. Cross-linking prevents the solubilization and possible loss of the aggregates after removing the precipitating agent [64]. This technique combines purification (precipitation) and immobilization (crosslinking) into a single operation and can be performed with crude preparations hence low costs are associated with it [103, 437, 446]. The precipitated aggregates are held together by non-covalent bonding without alteration of the enzyme structure and subsequent crosslinking makes the aggregates permanently insoluble while maintaining their pre-organized superstructure hence preserving catalytic activity [365, 447]. CLEAs demonstrate simple and low cost preparation, they are easy to recover and reuse, require no carrier, show high activity in organic solvents or unfavourable conditions, and are stable towards leaching in aqueous media [405, 448]. For example, Cabana *et al.* [64], produced *C. polyzona* laccase CLEAs using polyethylene glycol as precipitant and glutaraldehyde as a cross-linking agent. The CLEAs were used to eliminate EDCs such as nonylphenol, bisphenol A, and triclosan in a fluidized bed reactor and they displayed desirable thermal stability the cross-linking (presence of multiple links on the protein surface) prevented the unfolding of laccase under thermal stress conditions and removed all the EDCs at concentrations up to 5 mg/L. *T. versicolor* laccase CLEAs retained 80% of their activity after 70 days of storage at 4 °C and demonstrated 95% decolourisation efficiency for malachite green, bromothymol blue, and methyl red dyes [449]. *Cerrena sp.* laccase CLEAs demonstrated improved tolerance to NaCl, metal ions, and organic solvents [450]. Moreover, two or more enzymes can be precipitated simultaneously or sequentially to catalyze multiple biotransformation reactions [410].

Even though CLEAs show promises, their irregular shapes and sizes affect their recovery after reactions and diffusion of substrates to the active site [448]. Improvement of their properties has been studied and CLEAs of improved properties have been reported. For example, Nguyen *et al.* [448], synthesized hollow CLEAs of laccase (h-CLEAs) by employing a millifluidic reactor with two coaxial glass capillaries containing acetate buffer/laccase solution and acetonitrile/glutaraldehyde solution respectively. h-CLEAs were formed by rapid precipitation at the confluence zone where the two solutions met. Kumar *et al.* [451], reported synthesis of porous CLEAs through a three-phase partitioning technique where laccase and starch were co-precipitated, crosslinked with GA and the starch removed by α-amylase to create pores in the CLEAs. Entrapped CLEAs have been synthesized by using a combinatorial strategy of cross-linking and entrapping in mesoporous silica to form E-CLEAs [436]. Magnetic CLEAs formed through modification of CLEAs using functionalized and non-functionalized magnetic nanoparticles for increased activity, stability, and easy separation from the reaction mixture using a magnetic field have been reported [401, 452]. Laccase CLEAs have been extensively reviewed elsewhere [447].

Immobilization of individual enzyme molecules through covalent bonding to cross-linked polymer matrix to form single enzyme nanoparticles (SENs) has also been explored for laccase. For example, Şahutoğlu *et al.* [453], immobilized *T. versicolor* laccase as SENs via acryloyl chloride modification followed by in-situ polyacrylamide polymerization in water phase to form spherical 50 nm SENs. The multipoint enzyme attachment of the enzyme and the presence of the polymeric network enhance the conformational stability of the enzyme and the polymeric network is sufficiently porous to allow migration of the substrates to the active site [32].

7. Supports used for laccase immobilization

A suitable support material for enzyme immobilization should be compatible with the enzyme, inert in process solutions, and facilitate substrate diffusion to accomplish the biocatalytic reaction [85, 376]. Therefore factors such as volume, porosity, shape,

surface properties, stability in given reaction conditions, and cost are considered when selecting a support for immobilization [51]. For inert supports, their surfaces are modified to offer functional groups for protein binding [454]. Various materials have been explored over the years as supports for laccase enzyme (Figure 6, Table S2) [455]. The supports are classified into porous and nonporous supports (based on the structure) or organic and inorganic supports (based on their composition). Porous supports offer high enzyme loading with diffusion limitations especially for large molecular weight substrates [373] while nonporous supports have low diffusion limitations but suffer from low surface areas for enzyme immobilization [424]. Different materials have been explored as supports for immobilization of laccase enzyme (Table S2) and have been extensively discussed in this section.

7.1. Inorganic supports

Inorganic supports are reported to outperform organic materials in various aspects including microbial corrosion resistance, anti-swelling ability, mechanical and chemical stability, and better reusability [178, 249]. Various inorganic supports such as aluminium hydroxide [94], silica [17, 456], ceramics [362], Celite [457], perlite [423], and clay such as bentonite [129] and montmorillonite [342] have been utilized in immobilization of laccase.

7.1.1. Glass/silica

Silica has been widely used as a choice for immobilization because it is neutral, non-toxic, and occurs in the natural environment [188]. It is endowed with properties such as tunable particle size, porosity, surface area, transparency, chemical stability, and convenient preparation [22]. Various forms of silica including activated glass beads [249], perlite (amorphous volcanic glass) [423], porous silica beads [121, 458], silica gel [197, 365], controlled porosity glass/silica beads [26, 147, 210], and ordered mesoporous silica (OMS) such as SBA-15 [74,459] have been used for laccase immobilization.

In the past decade, much work has been undertaken in the synthesis of mesoporous silicates and in the immobilization of enzymes onto these supports. Mesoporous silica materials have attracted attention as catalyst supports due to their large specific surface area, mechanical stability, water insolubility, biocompatibility, adjustable pore size, highly ordered structure, and resistance to microbial attack [397, 406, 444]. More so, their surfaces can be chemically modified with various functional groups to enable electrostatic attraction between the support and enzyme while offering thermal and chemical stability [360, 365]. For example, functionalized ionic liquid supports were grafted onto mesoporous silica molecular sieves to obtain modified mesoporous silica with imidazole functional group for laccase adsorption [460]. Enzymes can be immobilized in mesoporous materials by different methods: adsorption, covalent attachment, or encapsulation.

The most important aspect when adsorbing enzymes on ordered mesoporous silica (OMS) materials is the pore opening diameter. Enzymes larger that the pore diameter cannot fit through the pore openings while pore opening way larger than the enzyme leads to enzyme leaching [461]. Encapsulation of enzymes in these supports renders them more mechanically robust and stable over time and covalent bonding is not required hence the native properties of the enzyme are maintained [305, 346]. Adsorption of the enzyme is suitable when the pore size is close to the enzyme dimension while covalent attachment is advisable when the pore sizes are far larger than the enzyme dimensions [462]. For large pore sizes, the silanol groups on the surface of the OMS can be activated with functional groups to strengthen the van der Waals interactions or serve as anchoring points for covalent immobilization [405, 461]. For example, Yang *et al.* [222], introduced amino functional groups in the pores of mesoporous silica using APTES and then crosslinked the activated carrier with GA prior to reaction with laccase solution.

7.1.2. Aluminium oxide

Aluminium oxide (Al_2O_3) is one of the metal oxides characterized with well-developed porous structures of varying pore diameters (ranging from 2 to 10 nm). It demonstrates high crystallinity, porosity, thermal, and mechanical stability [463]. Aluminium oxide in the form of pellets [234], mesoporous [463], nanoporous [464], and zeolite [198, 465] has been explored for laccase immobilization due to its high surface area, porosity, chemical, and mechanical stability. For example, Bruera *et al.* [464], adsorbed laccase on nanoporous aluminum oxide for treatment of black liquor. An immobilization yield of 18.1% was achieved and the biocatalyst retained 95.6% of its initial activity after consecutive runs and a 40 fold increase in removal of black liquor as compared to the free enzyme. Wehaidy *et al.* [466], immobilized laccase on nanoporus zeolite-x with an immobilization efficiency of 83% and 100% residual activity after 7 reuse cycles. When laccase was adsorbed in mesoporous aluminium oxide, the enzyme retained 65% and 60% of its initial activity after 30 days of storage and 10 reaction cycles respectively [463].

7.1.3. Nanoporous gold

Nanoporous gold (NPG) is a new type of porous inorganic material that has attracted attention in immobilization of enzymes due to its large surface area, its truly support-free, simple, and reproducible preparation methodology, tunable pore size, and clean nano-structured surfaces [368, 467]. It is prepared by etching alloys of gold and a less noble metal such as Ag, Sn, Cu, and Zn to form interconnected ligaments with gaps between them [468, 469, 470]. It is highly stable, highly conductive, has large surface areas, has easily functionalized nanostructured surfaces, and its pore sizes can be tuned from a few nanometers to several microns by adjusting the preparation method [469, 471]. It has been observed that enzymes confined within the NPG structure display stability to high temperatures and organic solvents due to the protective environment provide by the nanopores and biofouling can be greatly reduced since large proteins are restricted from accessing the internal pores [472]. For example, laccase was adsorbed on the surface of NPG and used in a biofuel cell for DET. The optimum temperature of the enzyme increased from 40 to 60 °C and highly efficient DET on a GCE was attained. Interestingly, the electrode showed no obvious changes in response after one month of storage at 4 °C [368].

Another advantage of NPG is that SAMs are more stable as they benefit from its defective sites, ligament surface, and lattice strain [472]. For example, Hakamada *et al.* [473], modified NPG with SAM of 4-aminothiophenol and used it for laccase immobilization. The SAM-modified NPG showed stronger bonding towards laccase and demonstrated higher thermal stability than the unmodified NPG.

7.1.4. Clay

The use of a natural supports such as clay or soil is desirable since it poses no environmental risk and is therefore beneficial for terrestrial bioremediation [126]. Clays are low-cost, eco-friendly, recyclable, have low mass transfer, demonstrate microbial corrosion resistance capacity, and their surfaces can be tuned through activation with desired functional groups and/or etching [474]. They present excellent properties such as high porosity, cationic exchange capacity, intercalation capacity, and swelling response [475, 476]. Various forms of clay such as bentonite [474, 475], sepiolite [477], smectite [476], kaolinite [77], and light expanded clay aggregates (LECA) [252, 478, 479] have been explored for laccase immobilization. For example, Dodor *et al.* [77], Immobilized *T. versicolor* laccase on kaolinite and used for the catalytic oxidation of PAHs, anthracene and benzo [a]pyrene, in the presence of ABTS as a mediator. The immobilized enzyme showed a broader optimum pH range of 4–6 as opposed to 4.5 of the free enzymes, storage stability of 100% activity after 4 months at 4 °C, stability in presence of sodium azide inhibitor, and increased the optimum operating temperature from 40 °C to 60 °C and 70% activity at 80 °C. Clay has also been mixed with other materials such as polymers and nanoparticles to improve on the performance of the biocatalyst. For example, Mehandia *et al.* [475], entrapped *Alcaligenes faecalis* laccase in chitosan-bentonite clay composite beads and achieved 88.4% immobilization yield with no leakage observed during washing or storage. Combinations of smectite nanoclay with carbon based nanomaterials (graphene oxide, carbon nanotubes and adamantylamine) were used for laccase immobilization with an immobilization yield of up to 85% and minimal alteration of the enzyme structure [476].

7.2. Organic supports

In this section, the various organic supports that have been explored for immobilization of laccase enzyme are discussed.

7.2.1. Carbon

Carbon has been explored as an enzyme support material due to is good mechanical strength, availability, easy modification with different functional groups such as carboxyl, amino, and phenolic-OH for enzyme coupling, and is already used in food and waste treatment industries [480]. Carboxylic functional groups can be easily formed on the surface of carbonaceous materials which provide ideal anchoring points for physical attachment and covalent bonding of enzymes on their surface [212]. Carbon can be obtained from wastes from the food industry hence provides cheaper alternatives for enzyme immobilization [150]. Various forms of carbon including graphite and its oxide [87], copper-containing ordered mesoporous carbon (Cu-OMC) [307], carbon fibers [481], magnetic bimodal mesoporous carbon [162], activated carbon [454, 480], and carbon nanotubes [482, 483, 484] have been used for laccase immobilization.

Carbon nanomaterials such as graphene oxide, SWNT, and MWNT are promising enzyme supports because of their chemical inertness, biocompatibility, and electrical conductivity [225, 381]. Graphene is a two dimensional monoatomic thick carbon material with high electrical and thermal conductivity, chemical stability, superior mechanical properties, nontoxicity, and high transparency usually derived from ordinary graphite [485, 486]. The two dimensional plate like structure of graphene offers a large surface area accompanied with incredible flexibility and great mechanical properties [289, 487]. Graphene offers low energy dynamics of electrons with atomic thickness, high carrier mobilities, abundant oxygen-containing functionalities, and water solubility making it an attractive support for laccase immobilization [224, 354]. The surfaces of graphene can be modified or derivatized to obtain congeners such as graphene oxide and reduced graphene oxide nanomaterials [485] and hydrogels or xerogels [488]. The use of graphene oxide and reduced graphene oxide to immobilize laccase have provided simple and less aggressive techniques [340, 377]. Due to its high hydrophilicity and tendency to agglomerate, graphene and its derivatives are combined with other nanomaterials such as Fe_3O_4 [382, 489,490], $CuFe_2O4$ [486], zeolite [491], PANI [338], MWNTs [492, 493], cellulose [494], zirconium-metal organic framework (Zr-MOF) [214], and Sb_2O5 [337] to aid its recovery. For example, Palanisamy *et al.* [339], adsorbed laccase on a screen printed electrode modified with graphene-cellulose microfiber composite for detection of catechol. A sensitive and selective biosensor was developed with a limit of detection of 85 nM, response time of 2s, linear response range up to 209.7 μM, and sensitivity of 0.932 μAμM−1 cm−2. Xu *et al.* [495], adsorbed laccase on a membrane developed by depositing GO nanosheets by layer-by-layer approach on a polyethersulfone support to obtain a biocatalyst with excellent stability towards temperature and pH over a long time.

Carbon nanotubes (CNTs) are nanowires based on tubular graphene layers of varying diameter, length, and chirality consisting of one (SWNT) or more (MWNT) nanotubes. They offer high adsorption capacity, large specific surface area, high enzyme loading, high conductivity, and they can easily be functionalized to obtain specific properties [496, 497]. CNTs easily bind to the enzyme non-covalently through hydrophobic interactions, formation of π–π stacks between enzyme residues and their surfaces, ampiphilic binding, electrostatic interactions, and hydrogen bonding between the enzyme amino groups and oxidized groups on their surfaces [285, 498]. For example, laccase was noncovalently immobilized on SWNT using a molecular tether (1-pyrene butanoic acid, succinimidylester) that formed an amide bond with the amine groups on laccase surface and coordinated to the SWNTs through π–π stacking with pyrene [499].

The unique tubular geometrical structure, superb electrical conductivity, high thermal conductivity, and superior tensile strength have made MWNT excellent enzyme carriers [176, 381]. MWNT can also be oxidized to produce carboxylated MWNT which have abundant oxygen-containing surface functional groups such as hydroxyl, carboxylic, and epoxy groups to enable the facile binding of

various molecules including proteins, enzymes, DNA, surfactants, and metals, etc., with or without coupling agents [482]. They have been utilized as electron mediators to facilitate electron transfer between electrodes and enzymes due to their high electrical conductivity [332, 344]. However, immobilization of laccase on MWNT has been reported to have limited impact on the thermal stability of the enzyme [498]. MWNT have been combined with other compounds such as cellulose nitrate, agarose, polyvinyl alcohol (PVA) [381], polynorbornene-pyrene [500], electrospun fibers [501], chitosan [334], mesoporous silica [502], poly (3,4-ethylenedioxythiophene) [335], nanoparticles such as Ni [317], Au [335], and anthraquinone moieties [290, 503] to increase their stability through reduced agglomeration for continuous or multibatch processes. For example, laccase was adsorbed on a MWNT/Ag-nZnO composite by dropcasting laccase on a screen printed electrode modified with MWNTs that are functionalized with silver doped zinc oxide nanoparticles (nAg-ZnO) for detection of bisphenol A [331].

7.2.2. Agricultural waste

Use of synthetic support materials can not only be expensive but also present a concern for their disposal. As a result investigations focused on easy to find, inexpensive, biodegradable, and easily functionalized supports have led to the adaptation of agro-industrial wastes as enzyme supports [28]. Various agricultural products and wastes including eggshell membranes [504], coconut fiber [269, 505], *Luffa cylindrica* fiber [209], chicken feathers [506], pallet wood [507], pistachio nut shell [508], maize starch [228], spent grain [509, 510], biochar [181], and bacteria [511] have been used for laccase immobilization. For example, Montoya *et al.* [150], produced mesoporous carbon supports from various wastes, that is, pecan nut shells, peach stones, pistachio shells, and pine nut shells using activating agents to promote mesoporosity. Cristóvão *et al.* [391], adsorbed laccase on green coconut fibre for dye decolourisation and obtained an immobilization yield of up to 69% and recovery yield of up to 45%. Da silva *et al.* [512], immobilized laccase on beer spent grain (SG) and digested beer spent grain (DSG) by adsorption and covalent bonding through modification of DSG of the supports with glycidol and glycidol followed by ethylenediamine. The DSG was obtained by acid/base treatment of SG. Modification of the supports to obtain covalent immobilization significantly decreased the laccase activity and immobilization yield. A high immobilization yield of up to 90% was obtained through adsorption and the half-life of laccase increased from 0.64 to 1.1 h at 70 °C. Nanocellulose for laccase immobilization has also been synthesized from wastes such as quinoa husks [247] and sugar mill bagasse.

Biochar is a carbonaceous material obtained from hydrothermal and thermochemical methods of biomass conversion and offers as a large specific surface area, good dispersibility, and biocompatibility for stable and high-load enzyme immobilization [513, 514, 515]. The raw materials used for its production and the production conditions affect the chemical and physical properties of the biochar produced and hence its utilization in enzyme immobilization [516]. In addition to being inexpensive and easily available, these methods also provides the potential for waste valorization [426]. For example, Naghdi *et al.* [212], immobilized laccase on pinewood nanobiochar functionalized through acidic treatment (HCl, H_2SO_4 and HNO_3). When laccase was covalently immobilized on citric acid pretreated micro biochar from pine wood, pig manure, and almond shell using glutaraldehyde, it was observed that feed stock selection and method of production affected biochar properties (texture, morphology, surface area and cationic nature) and consequently the immobilization efficiency [207, 426]. Since the enzyme immobilization efficiency was dependent on ionic strength and cationic nature of the biochar, biochar from pig manure offered the best support due to its high cationic exchange capacity compared to the wood based biochar [426]. Immobilization of laccase on biochar has been extensively reviewed elsewhere [514, 517].

7.2.3. Polymers

Polymer based carriers have attracted attention in enzyme immobilization because of their easy surface modification where various reactive groups can be anchored on the matrix and their morphology can be tuned during the polymerization step [379]. Properties such as surface area, functional group density, and porosity can be tailored to meet the specific needs for immobilization [389]. A variety of polymeric support materials including natural (chitin, chitosan, agarose, and cellulose derivatives) and synthetic (nylon, polysiloxane, polyvinyl alcohol [431], poly (glycidyl methacrylate), and polyacrylic polymers) have been prepared in various geometric forms and used for the immobilization of laccase. Biological or natural polymers have demonstrated advantages such as low cost, nontoxicity, and biocompatibility with enzymes through interaction with their functional groups [122]. However these supports suffer from low mechanical strength and are liable to microbial degradation necessitating the need for synthetic polymers [245]. The various polymers that have been utilized in laccase immobilization are discussed in this section.

7.2.3.1. Natural polymers. Various natural polymers have been used as supports for laccase immobilization and have been discussed in the following section [518].

7.2.3.1.1. Chitosan. Chitosan (CS) is a partially deacetylated polymer that is obtain via alkaline deacetylation of chitin [129]. Chitin is a biodegradable and non-toxic polysaccharide mainly obtained from marine and terrestrial invertebrates [366, 519]. The degree of deacetylation, concentration, and molecular mass greatly determine the solubility of CS and in turn the properties of the enzyme conjugates [520]. CS is endowed with attractive properties including hydrophilicity, biodegradability, biocompatibility, nontoxicity, and many large amine groups [314, 318, 399]. In addition, CS has demonstrated resistance to microbial degradation compared to other organic supports and is obtained from chitin, the second most abundant natural material on earth after cellulose [521]. This implies that CS is widely commercially available at low cost making it an ideal carrier for enzymes [25, 522]. The amine and OH groups on its surface give it excellent affinity to a variety of enzymes and solubility in mildly acidic aqueous solutions making it a good immobilization support [421, 523]. The presence of amino and OH functional groups on chitosan have allowed it to be used as a coating on other enzyme supports such as alginate beads [235] to improve their biocompatibility with laccase [345]. For example, during the entrapment of *P. ostreatus* laccase on copper alginate beads, enzyme retention during dye decolourisation was greatly

improved when the copper alginate beads were coated with chitosan [524]. Immobilization of enzymes on CS has been shown to protect the enzyme from metal ion inhibitors [359]. The polymer has been used in various forms and shapes such as powder [525], membranes [526], beads [67, 404, 527] and hydrogel [528, 529] for immobilization of laccase.

Due to its soft texture, CS is usually cross linked with coupling agents such as GA to minimize dissolution in acidic conditions or incorporated with organic or inorganic materials to improve it mechanical stability and immobilization efficiency [28, 129]. For example, Cu (II)-chelated chitosan-graft-poly (glycidyl methacrylate) nanoparticles were prepared using poly (ethylene imine) (PEI) which acted as a spacer arm and metal chelator for laccase immobilization by coordination. The high density of amino functional groups on PEI provided the ideal conditions for chelating metal ions [521]. Addition of bentonite clay laccase solution to CS to form CS-clay laccase composite beads improved the density, surface area, pore volume, and pore size of the support hence yielding improved reusability, thermal-, and operational stability of the biocatalyst [129]. Laccase immobilized on glutaraldehyde-activated chitosan [170], CS/CeO2 microspheres [523], itaconic acid grafted and Cu(II) ion chelated CS membrane [526], CS coated magnetic nanoparticles [192], Alginate/CS microspheres [40], N-doped carbon hollow spheres/CS composite [315], MWNT-CS [345], halloysite nanotubes (HNTs)/CS microspheres [530], HNTs/Fe_3O_4/CS composite [179], magnetic multi-walled carbon nanotube-chitosan/silica hybrid membranes [333], and polyethyleneimine grafted chitosan films [531] has been reported.

7.2.3.1.2. Cellulose. Cellulose is the most abundant natural polymer and is nontoxic, biodegradable, hydrophilic, and biocompatible making it an excellent support for enzyme immobilization [313]. In addition, its derivatives such as cellulose acetate, cellulose nitrate, and carboxymethyl cellulose also offer excellent biocompatibility [168, 532]. Cellulose usually varies in morphology, size, crystallinity, and surface functional groups depending on the source of agricultural waste used during synthesis [247]. Laccase has been immobilized on cellulose based carriers such as cellulose acetate [313], nanocrystals [533], cellulose based Granocel carriers [8, 379] and solubilized cellulose, and PAMAM films [534]. For example, Ghodake *et al.* [211], extracted α-cellulose fibers form waste-paper biomass and super-magnetized them with Fe_3O_4 followed by grafting with CS and thiol groups for laccase immobilization.

Even though most cellulose has been isolated from plants, several bacteria from the genus *Gluconacetobacter* have recently been reported to produce a unique form of cellulose commonly called bacterial cellulose (BC) [535]. BC has demonstrated excellent characteristics such as tridimensional and fibrillar nanostructure, high water absorption capacity, and excellent mechanical strength which differ from plant cellulose [535, 536]. Since BC is nontoxic, biocompatible, bioavailable, non-carcinogenic, and easily biodegradable, it has been explored in enzyme immobilization for biomedical applications such as wound dressing, burn treatment, and tissue regeneration [284]. Due to its fibrillar nanostructure, large surface area, and high porosity, BC is expected to easily entrap enzymes and achieve high enzyme immobilization yield and efficiency [536]. For example, Li *et al.* [324], hybridized BC nanofibers by depositing gold nanoparticles (AuNPs). Laccase was then adsorbed to the hybrid followed by addition of Nafion. The biocatalyst was used as a biosensor for detection hydroquinone with a low detection limit of 5.71 nM and a wide linear range of 30–100 nM. Laccase adsorbed on BC aerogels for degradation organic dyes degraded 94.5% and 85.2% of reactive red and 2,4 dichlorophenol respectively within 4 h and retained 76% activity after 5 reuse cycles [537].

Enzyme immobilization on cellulose supports is through simple physical adsorption or weak inter ionic interactions between the enzyme and activated hydroxyl groups on cellulose [534]. Drozd *et al.* [538], reported that exposing BC to a rotating magnetic field prior to laccase immobilization improved the thermal stability of the biocatalyst by 10 °C and activity retention after 8 cycle by 15%.

7.2.3.1.3. Agarose/sepharose. Agarose based gels are used as matrices for enzyme immobilization due to their biocompatibility, hydrophilicity, and presence of hydroxyl groups on the surface that can be activated for covalent bonding with enzymes [72, 539]. For example, Brugnari *et al.* [540], immobilized *P. ostreatus* laccase on MANAE-agarose for degradation of bisphenol A. An immobilization yield of 100% and 138% activity of the immobilized enzyme compared to its free form were obtained. The biocatalyst also demonstrated thermal and storage stability, longer half-life and retained 90% of its initial activity after 15 reuse cycles. Laccase immobilized in sepharose-CL-6B glass beads has also showed great stability in organic solvents such as hexane, tetrahydrofuran, and dioxane [539]. Agarose activated with functional groups such as hydroquinone and β-benzoquinone [10], glyoxal [541], thiolsulfinate [542], aldehydes [165], and anti-laccase antibodies [253] have been explored for laccase immobilization.

7.2.3.1.4. Alginate. Alginate is a natural polymer easily converted to hydrogel via crosslinking its carboxyl groups with solutions of cations such as Ca^{2+}, Ba^{2+}, and Cu^{2+} [115, 157]. The polymer is nontoxic and biodegradable and entrapment in alginate beads is one of the cheapest, fastest, and simplest methods to develop efficient and stable laccase [137, 250]. Laccase has been entrapped in various alginate beads such as calcium alginate–chitosan beads and sol–gel matrices [122], Calcium alginate beads [115], and copper alginate beads. For example, Domínguez *et al.* [136], entrapped *T. hirsuta laccase* in calcium alginate beads for decolourisation of indigo carmine and phenol red in an airlift bioreactor. 96% and 69% decolourisation efficiency were achieved for indigo carmine and phenol red, respectively, after 24 h and the bioreactor operated uninterrupted for 40 days without any significant changes in its activity. The bead sizes allowed movement of air bubbles throughout the reactor bed giving suitable aeration for the microorganism and avoiding clogging problems which would hinder mass and oxygen transfer rates. Niladevi and Prema, 2008 [157] entrapped *Streptomyces psammoticus* laccase on Ca and Cu-alginate beads and Cu-alginate provided higher immobilization yield (61%) than Ca-alginate (42%). This was because Cu has a higher affinity for alginate than Ca and strongly complexes within the polymer network. The Ca-alginate beads also had higher porosity and lower chemical stability leading to higher enzyme leakage than Cu-alginate beads. However, Lassouane *et al.* [543], observed that crosslinking of laccase with GA prior to entrapment in Ca-alginate beads increased the immobilization yield by 30% and reduced leakage by 7 fold.

7.2.3.1.5. Gelatin. Gelatin has abundant free amino, hydroxyl, and carboxyl functions in molecule chains and GA can interact with free amino groups in gelatin or the enzymes, which makes water easily exude from the beads during the crosslinking and prevents the leakage of immobilized enzymes from the beads. Owing to its low intensity and high brittleness, gelatin is rarely used alone and is often used after crosslinking, grafting, or blending [544].

7.2.3.2. Synthetic polymers. Various synthetic polymers such as Polyacrylonitrile [183, 545], polyethersulfone [546], Polypropylene [190], polyamide [376], polyhydroxybutyrate [547], polyurea [548], poly (ethyleneimine) [350], poly (ethylene terephthalate) (PET) [432], polypyrrole [68], polypropylene chloride [549], polymethacrylate [229], and Polyacrylamide gel [18] have been used for immobilization of laccase. The most commonly used synthetic polymers for laccase immobilization are discussed in this section.

7.2.3.2.1. Eupergit. Eupergit is an epoxy activated polymer that binds to enzymes via epoxide groups [172, 550]. Its oxirane groups function as active components for covalent binding of ligands containing amino, mercapto, or hydroxyl groups [48, 551]. Due to their hydrophilicity, wide pore distribution, and good hydrodynamic properties, they offer a simple coupling procedure to the enzymes [552]. Hublik and Schinner [48] immobilized *P. ostreatus* laccase on Eupergit C. benzoate and used the bioconjugate for continuous elimination of 2, 6-dimethoxyphenol in a packed bed reactor. No substrate was detected in the filtrate and the bioconjugate showed increased activity and great stability with a 2% registered loss after 10 days. When laccase-eupergit bioconjugate was used in a fluidized bed reactor for the removal of estrogens, prolonged enzyme stability of over 16 days was attained [553].

7.2.3.2.2. Polyaniline. Polyaniline, a conducting polymer, has been explored for laccase immobilization particularly for biosensor and biofuel cell applications [311]. It is environmentally safe, can be cheaply manufactured, can act as a direct mediator for electron transfer in redox reactions, and exhibits high conductivity and thermal stability [330]. For instance, Mani *et al.* [554], compared the catalytic oxygen reduction in microbial fuel cell of laccase immobilized by crosslinking with PANI, entrapment in Cu-alginate, and encapsulation in Nafion micelles. The PANI-laccase showed the highest stability, activity, and reusability. PANI has been used in various forms such as films [159], nanofibers [330], and printable ink [555] for laccase immobilization. For example, Timur *et al.* [159], adsorbed *T. versicolor* laccase on a PANI modified thick film electrode and used it as a biosensor for detection of phenol, catechol, and $_{L}$-DOPA. Laccase was also immobilized on porous PANI nanofibers in a three-step process of enzyme adsorption, precipitation, and crosslinking for detection of phenolic compounds. The biocatalyst retained 100% of its initial activity after 6 days of storage at room temperature and 74 after 3 h of incubation at 60 °C [556]. When the biocatalyst was used as a cathode in an ABTS mediated biofuel cell with glucose oxidase-PANI nanofibers as the anode, the biofuel cell demonstrated a power density of 37.4 $\mu W/cm^2$ and retained 54% and 70% of its initial power density after 28 days of storage at room temperature and 4 °C, respectively. PANI has been combined with other compounds such as poly (methyl methacrylate) [557], magnetic graphene [338], MWNTs [558], graphene oxide [559], and cellulose [329].

7.2.3.2.3. Poly (vinyl alcohol) (PVA). PVA based materials have been used to entrap enzymes especially for biosensor applications due to their low cost, stability, recyclability, biocompatibility, and easy manipulation with different activation methods for enzyme immobilization [377, 560, 561]. Also PVA polymers are reported to have high mechanical strength which is an important attribute for an enzyme support [118]. Different forms of PVA such as particles [372], beads [118], cryogels [431], hydrogels [562, 563], and microspheres [560] have been used to entrap laccase. For example, Gonzalez-Coronel *et al.* [434], entrapped laccase in Lentikats (lentil shaped particles of PVA) and obtained 89% activity recovery, 0% leaching, and 90% retained activity after 84 h. Laccase entrapped in PVA microspheres demonstrated stability on organic solvents (methanol, ethanol, acetone, and DMSO) for concentrations up to 40% [560]. However, PVA is susceptible to deformation at higher temperatures hence the biocatalyst can only be used for low temperature reactions [560]. To overcome that, PVA has been combined with other supports such as halloysite nanotubes [564], metal organic frameworks [561], alginate [377], xylan [565] and mesoporous silica [328].

7.2.3.2.4. Nafion. Nafion is a polyanion with a hydrophobic fluorocarbon backbone and hydrophilic cation-exchange sites making it moderately hydrophobic [348, 566]. It is usually used in biosensors and biofuel cells to provide a high adsorption for laccase and to prevent enzyme leaching from the electrode surface as well as undesirable side reactions [567, 568, 569]. Nafion is preferred in biosensors because its negatively charged polyelectrolyte matrix eliminates the influence of interfering signals hence improving the sensitivity of the sensor [323]. For example, Litescu *et al.* [327], drop-casted a laccase solution on a screen printed electrode, allowed it to dry with forced air followed by immobilization of a Nafion membrane to protect the enzyme from leaching. Đurđić *et al.* [325], drop-coated laccase on a screen printed carbon electrode modified with graphene nanoplates @MnO_2 nanoparticles, followed by drop-coating of Nafion solution and allowing to dry. Nafion has been reported to improve the long-term stability, enhance the adhesion and binding force, and the selectivity of a biosensor [326]. Choi *et al.* [570], dispersed MWNTs in Nafion solution to form MWNTs/Nafion composite which was then used to immobilize laccase covalently using EDC/NHS. The MWNTs/Nafion nanocomposite provided a microporous structure with a large surface area for laccase immobilization.

7.2.3.2.5. Mixtures and hybrids of polymers. Because polymers suffer from low mechanical strength and ease of microbial attack, new techniques have been developed to enhance their mechanical properties such as crosslinking, mixing the polymers with other polymers or inorganic materials [198], graft copolymerization, and use of interpenetrating polymer networks (IPNs). Supports of polymers mixed with other materials such as Alginate-carbon beads [571], polyacrylamide-alginate cryogels [572], alginate-gelatin Mixed Gel [162], polyvinyl-alginate-silicon dioxide matrix [130], poly (amido amine) (PAMAM) dendrimers [534], gelatin blended with polyethylene glycol [544], butyl acrylate, and ethylene glycol [390] have been reported for laccase immobilization with improved immobilization yields and efficiencies compared to the supports from polymers alone. Graft copolymerization is the addition of functional groups to a polymer through covalent bonding of monomers onto the polymer chain to improve its mechanical properties and stability [521, 546]. The properties of the resultant copolymer combine properties of the prepolymer and the grafted molecule and these properties can be regulated by varying the concentrations of initiators, crosslinkers, and the prepolymer [573]. IPNs are combinations of two polymers in network form in which one polymer is synthesized and/or crosslinked in the presence of another [245, 402].

Smart polymers have also been utilized to immobilize laccase with the hope of retaining its activity over a wider range of pH, temperature, and ionic strength [365]. For example, Klis *et al.* [321], covalently immobilized laccase on poly (N-isopropyacrylamide) gel attached to a dimethoxyloxyvinylsilnae silanized ITO electrode, and determined its redox and catalytic properties at varying sizes

of the gel matrix. It was observed that the catalytic efficiency decreased with shrinking gel size but the enzyme structure remained unchanged.

Crosslinking of polymers to form gels (hydrogels and cryogels) for entrapment of laccase has also been reported as the gels present improved resistance to thermal and chemical inactivation, remarkable storage, and operational stability [573]. For example, laccase was covalently immobilized on a composite of methyl methacrylate (MMA)/butyl acrylate (BA) copolymer latexes, and rGO hydrogels. The composite hydrogels were synthesized by self-assembly of graphene oxide nanoparticles in the polymer latex matrix where the polymer nanoparticles were adsorbed on the rGO surface [487]. Hydrogels are soft and highly hydrated polymeric networks synthesized via covalent or ionic crosslinking of monomers with or without metal ions [574]. Entrapment of enzymes in hydrogel leads to secondary interactions between the enzyme and the polymeric matrix which may cause a shift of the enzyme maximum activity to a higher pH than the free enzyme [402]. And since the retention and expression of enzyme activity are closely related to the particle size, porosity, and polarity of the supporting matrix, the structure of the hydrogel needs to be optimized for maximum enzyme activity [573]. Electron-beam grafting techniques have also been explored to form gels in the absence of initiators and crosslinkers. For example, Jahangiri *et al.* [575], used electron beam irradiation on a mixture of poly (vinylidene fluoride) membranes and laccase to form membrane-laccase bioreactors and on a mixture of macroporous polymeric gels, laccase, syringaldehyde, and ABTS to form cryogel bioreactors for degradation of bisphenol A. By using high-energy electron radiation cryogels can be synthesized without additional initiators or cross-linkers within 10 min (excl. freezing time) as larger bulk materials allowing furthermore the simultaneous incorporation of macromolecules in a one-step process.

7.3. Electrodes

Laccase has been immobilized on electrodes for application in biosensors and biofuel cells [3]. An immobilization strategy that maximizes coverage of enzyme on the electrode surface with high operational stability and ensures maximum electron transfer between the redox sites of the enzyme and the electrode surface and minimum loss of enzyme from the electrode surface through leaching are the most important aspects considered during immobilization [301, 344]. Therefore the development of biosensors based on enzyme modified electrode surfaces in such a way that the enzymes' full activity and stability are retained has become an important aspect in various fields ranging from environmental analysis to clinical diagnosis [343, 481]. Immobilization of enzymes on electrode surfaces should ensure that the redox centres of the enzyme are as close as possible to the electrode surface to allow rapid electron transfer between the enzyme and electrode [101, 322]. The immobilization method is also an important factor as it should provide proper orientation of the enzyme efficient electronic contact with the electrode [576].

Various immobilization techniques such as physical adsorption and covalent immobilization have been explored. For example, Haghighi *et al.* [87], adsorbed *T. versicolor* laccase on spectrographic graphite electrode for use in an amperometric flow through cell to monitor phenolic compounds in a single line flow injection system. However, when enzymes are directly adsorbed on electrodes, there is poor electronic communication between the enzyme redox centers and the electrode surface hence electron mediators that can shuttle electrons between the enzyme and electrode conductive surface are added [39, 292, 576]. Chemical modification of electrode surfaces with appropriate functional groups for covalent linkage has also been explored [44, 577]. For example, Quan *et al.* [46], covalently immobilized laccase on silane modified platinum electrode surface and used it as a biosensor for ABTS, p-phenylenediamine (PPD), and p-aminophenol (PAP). The sensor gave a response time of 2 s for PPD and PAP and 6 s for ABTS. When the pH of the media was changed, the redox potentials for PPD and PAP changed by 60 mV per 1 unit of pH and no change in redox behaviour for ABTS was observed. Freire *et al.* [481], immobilized laccase on carbon-fibre electrodes using physical adsorption, GA, carbodiimide, and carbodiimide/GA approaches. The highest biosensor activity was obtained using carbodiimide/GA for coupling laccase to carboxyl groups on carbon fibres.

Entrapping laccase in other materials including Montmorillonite [342], Nafion [164], and PANI [318] is utilized to prevent leaching of the enzyme into the solution and cracking of the enzyme matrix. For example Li *et al.* [578], entrapped laccase in Poly (3, 4-Ethylenedioxythiophene) (PEDOT) on a Pt electrode through electropolymerization of EDOT and laccase solution from 0.2 to 1.3 V at a scan rate of 100 mV/s. Rochefort *et al.* [322], used interfacial condensation of polyethyleneimine (PEI) to prepare microcapsules containing laccase. The laccase was mixed with PEI in an aqueous phase that was emulsified in an organic phase prior to addition of a water-insoluble crosslinking agent. The microcapsules were adsorbed onto a GCE and the charged micro-environment offered by the microcapsules led to a shift in optimum pH and half saturation constant (K_m) of the enzyme and storage stability of 73% activity after six months of storage. Palys *et al.* [579], entrapped laccase in poly-o-phenylenediamine (POPDA) on a GCE and the POPDA acted as an immobilizing matrix as well as a redox mediator during reduction of molecular oxygen. This approach displayed minimal distortion of the three-dimensional structure of the enzyme and gave comparable current density values to those obtained when other mediators were used and higher values compared to mediator-less electrode systems.

For efficient contact between the enzyme and electrodes, mediators are sometimes added and are either dissolved in the analyte solution or co-immobilized together with the enzyme [580, 581]. For example, Nogala *et al.* [582], co-immobilized laccase and ABTS on a silicate carbon ceramic electrode for biolectrocatalytic reduction of dioxygen. This allowed close contact of the mediator and enzyme hence efficient electro transfer and electrocatalysis was achieved. However, the electrode suffered immensely from laccase deactivation by ABTS radical. Brunel *et al.* [292], entrapped laccase and ABTS on polypyrrole polymer through electropolymerization by controlled-potential electrolysis in the presence of a mixture of biomolecules and monomer solution and used it as a cathode in a glucose/O_2 biofuel cell. When laccase was immobilized on mesoporous silica (MCM-41) support modified with methylene blue dye (MB) as a mediator and used as a biosensor for detection of phenols, the MB-MCM-41 modified electrode displayed a shorter response time (less than 4s) than the MCM-41 modified electrode without MB as a mediator (15 s) [346]. Laccase-mediator co-immobilized

bioconjugates have been reviewed elsewhere [583]. The advantages of systems with co-immobilized mediators are that the rate of reaction is not limited by the mediator diffusion leading to higher bioelectrocatalytic current values, there is minimal pollution from residual enzyme and mediator in solution, an appropriate choice of mediator can be used to tune the potential of the reaction, and there is facilitated recovery and reuse of the mediator [579, 580, 584]. However, drawbacks such as high cost, irreversible oxidation/reduction of mediators, leaching into the reaction system, and sometimes generation of toxic byproducts have to be considered prior to selection of a mediator [575].

Other than electrodes, laccase has been explored in bioactive paper. For example, Savolainen *et al.* [585], entrapped *Trametes* sp. laccase in PEI microcapsules via interfacial polycondesation of PEI with sebacoyl chloride cross linker. The microcapsules were incorporated into an ink and coated on paper substrate to form bioactive paper. A similar approach was utilized by Guerrero *et al.* [407], and in both cases, it was observed that the use of microencapsulation allows better activity retention of laccase on papers over time at room temperature and decrease the rate of laccase inhibition in presence of inhibitors.

7.4. Metal organic frameworks (MOFs)

MOFs are mesoporous structures consisting of metal ions or clusters coordinated to organic ligands [365]. They are synthesized by diffusion of a combination of metals and organic skeletons [586]. They are associated with ultrahigh and tuneable porosities, large surface areas, multiple functional sites, structural flexibility, chemical, and thermal stability [587]. More so, their organic bridging ligand can be synthetically modified to introduce desired functional groups to the framework [588, 589]. MOFs have been synthesized with various metals and metal oxides such as copper [586], iron [590], zirconium [588], and zeolite [587] for laccase immobilization. Ladole *et al.* [591], reported synthesis of magnetic MOFs (MMOFs) consisting of peroxidase and magnetic nanoparticles. Laccase was embedded in the MMOFs to form a biocatalyst of 100 nm average diameter, high thermal stability, and recyclability of 89% residual activity after 10 cycles, and storage stability of 100% residual activity after 30 days. Entrapment of laccase in nanocrystalline NH_2-MIL-53 (Al) demonstrated high enzyme loading, permanent retention of the enzyme and stability. The bioconjugate was used for the removal of bisphenol A from water and it achieved 100% removal after 3 min [220]. Immobilization of laccase on MOFs has been extensively reviewed elsewhere [592].

7.5. Nanomaterials

Various inorganic and organic nanomaterials such as silica nanoparticles [593], Manganese ferrite nanoparticles [594], copper ferrite nanoparticles [595], Titanium oxide nanoparticles [256], *Halloysite nanotubes* (HNTs) [178, 295], *Graphene nanosheets* [110], ZnO nanoparticles [596], carbon nanotubes and nanospheres [206, 310, 415], gold nanoparticles, and metal oxide nanoparticles have been used as carriers to immobilize enzymes. The advantages associated with immobilizing enzymes on nanomaterials are: they provide high surface area for enzyme attachment and high effective enzyme loading, high mechanical resistance, and their similar size to enzymes ensures minimal diffusion limitations and a reduced mass transfer resistance [75, 192, 597]. Therefore, they provide an ideal solution to the challenges encountered in optimization of enzyme immobilization on nonporous supports and provide a transition between homogenous and heterogeneous catalysis [398]. These nanomaterial are available in different sizes, shapes and compositions, and retain their characteristics after functional activation even when very harsh chemical modifications are carried out [386]. More so, when combined with enzymes, they do not interfere with the active site hence preserving the biological activity of the enzyme [412].

Magnetic nanoparticles (MNPs) have been widely studied due to their superparamagnetism and ease of separation from the reaction mixture using an applied magnetic field [192, 433, 598]. This improves their reutilization since there is no need to for energy and time consuming centrifugation and filtration steps and the enzymes are subjected to very low mechanical stress compared to when nonmagnetic nanomaterials are used [400, 414, 599]. Nevertheless, bare magnetic nanoparticles are highly susceptible to acidic and oxidative conditions and when directly exposed to the biological system, they influence the stability and activity of the enzyme [227, 446, 600]. To improve their functionality, they are entrapped in other compounds such as chitosan [184, 446], carbon [227], PEI [302, 414], and graphene oxide [422] and then functionalized for enzyme bonding. For example, Kalkan *et al.* [599], coated Fe_3O_4 (magnetic) nanoparticles with chitosan followed by activating the hydroxyl groups of chitosan with carbodiimide or cyanuric chloride for covalent binding with laccase. Fernandes *et al.* [75], functionalized silica-coated magnetite NPs with (trimethoxysilylpropyl)ethylenediamine triacetic acid (EDTA-TMS) and chelated with copper (II) ions as a metal ligand for laccase immobilization. PEI modified Fe_3O_4 nanoparticles (Fe_3O_4–NH_2–PEI NPs) were chelated with Cu^{2+} to immobilize laccase through metal affinity adsorption [414].

Silica based nanoparticles are also highly suitable for enzyme immobilization due to their environmental friendliness, high biocompatibility, and resistance towards organic solvents and microbial attacks [386, 593]. Silica based nanoparticles have been widely reported for immobilization of laccase enzyme [74, 114, 188, 281, 462]. Laccase was immobilized by sorption on amino-modified silica nanoparticles with subsequent covalent crosslinking using glutaraldehyde. An immobilization efficiency of 98.2% was obtained and subsequently retained 63% activity after 32 days incubation in phosphate buffer at pH 7 and 77% after 25 days incubation in real waste water [158].

Electrospun nanofibers from polymeric materials such as cellulose [168], poly (ε-caprolactone) [601], polyacrylonitrile [251], and Poly (lactic-co-glycolic acid) [175] have gained attention as well due to their large surface area, high porosity biocompatibility with tissue and cells, and a subcellular size that makes them good carriers for enzymes [521, 602]. These nanofibers offer minimal mass transfer limitations, low environmental risk, and their surface and texture can be modified to suit a specific enzyme [191, 200]. Zdarta *et al*, [205] immobilized laccase on poly (methyl methacrylate) (PMMA)/Fe_3O_4 electrospun nanofibers through covalent binding and

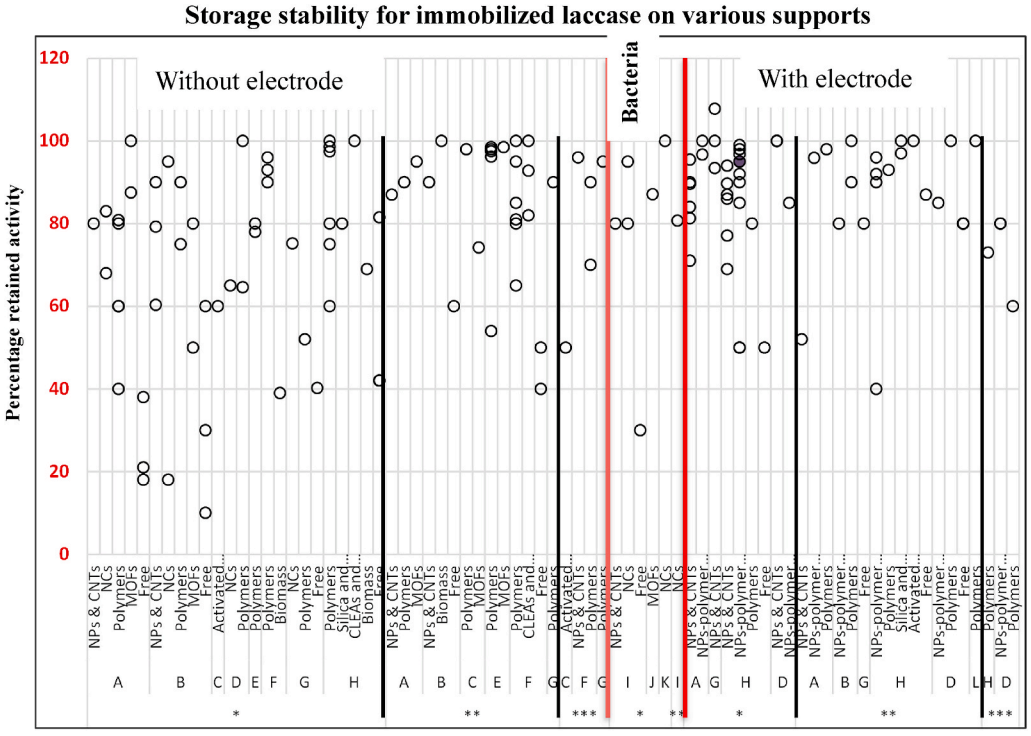

Figure 7. A graph showing the storage stability (percentage retained activity after storage) of free and immobilized laccase on various supports after 1 month, 1–4 months and over 4 months.

entrapment. The biocatalyst exhibited excellent pH, thermal and storage stability, and high enzyme loading and reusability where 80% activity was retained after 40 days of storage and 5 successive biocatalytic cycles.

Composites consisting of two or more nanomaterials have also been studied for laccase immobilization. For example, Poly (hydroxyethyl methacrylate-N-methacryloly-(l)-histidinemethylester)-copper (II) [PHEMAH-Cu^{2+}] nanospheres [398], TiO_2--Montmorillonite (MT) complexes [385], Fe_3O_4/SiO_2 nanoparticles [230, 231], Polyacrylonitrile-biochar composite nanofibrous membrane [180], SWNT/nanocrystalline carbon quantum dots [336], CS/PVA composite nanofibrous membranes [191], ZnO/SiO_2 nano-composited [243], Zinc tetraaminophthalocyanine-Fe_3O_4 nanoparticle composite [394], and copper tetra-aminophthalocyanine (CuTAPc)-Fe_3O_4 [296,392,603].

Nonmagnetic nanoparticles have been combined with other processes to improve their reusability especially in waste water treatment [597]. For example titanium oxide nanoparticles have been mixed with polyvinylidene fluoride (PVDF) [169] and polyethersulfone (PES) membranes [597]. Cao *et al.* [428], coated a polydopamine/gold nanoparticles hybrid layer on the surface of silica shell encapsulated n-docosane through surfactant-assisted self-assembly and in-situ reduction of Au ions to form self-thermoregulatory microcapsules for laccase immobilization.

As shown in Figure 7, immobilization of laccase on supports improves its storage stability as immobilized laccase generally retains over 80% as compared to free laccase that retains less than 40% over time. Most of the laccase completely lost its activity within a period of 4 months (not shown on the graph). It is noteworthy that further immobilization of the biocatalyst on electrodes further enhances the storage stability with retained activities of over 80% for over 4 months. NPs and NCs facilitate high enzyme activity retention regardless of the laccase source even for periods of over 4 months. This could be due to their small size that allows minimal diffusion limitations hence enabling the enzyme to behave as if it were free and at the same time with the protection from denaturation by the polymer.

On top of storage stability, the reusability of the biocatalyst also has to be considered for industrial applications. Although polymers provide favourable storage stability over time, the reusability is limited to a few cycles (less than 50). This can be improved by impregnating the polymers with metal NPs to form metal-polymer nanocomposites to allow free expression of the enzyme with maximum protection from the polymer. This is evident in Figure 8 as the NCs and NPs supports allow reusability for the enzyme retaining high activity for over 50 cycles. Furthermore, immobilization of the supports on electrodes shows remarkable retained activities for more cycles. For industrial applications, a support matrix that retains the enzyme activity, allows good storage stability and reusability for longer periods of time is desirable and all these factors need to be put into consideration when selecting an immobilization support.

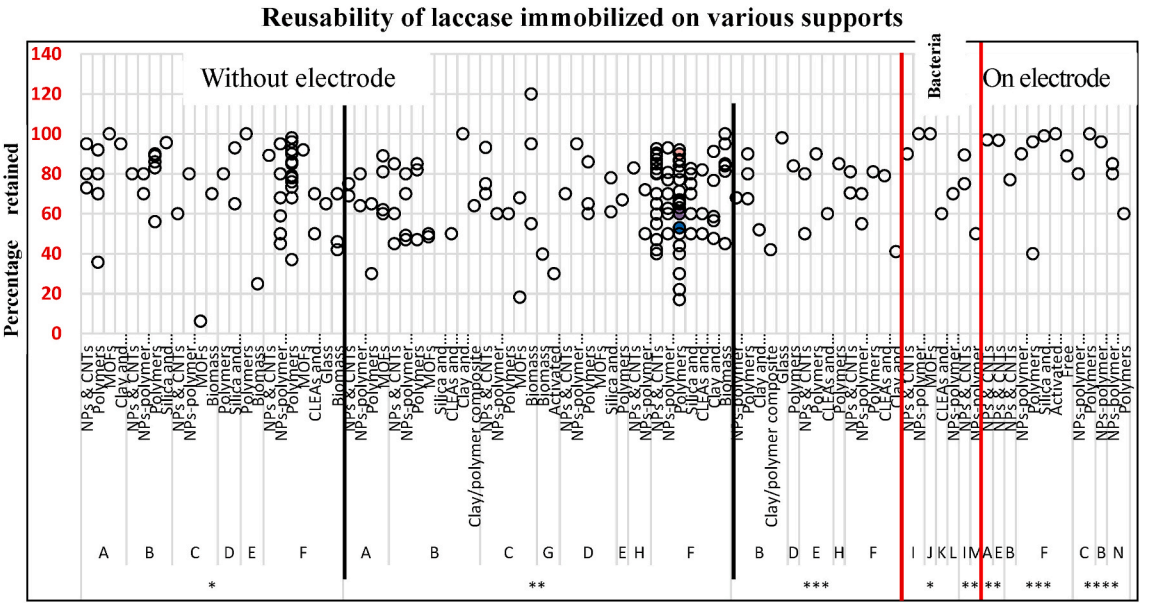

Figure 8. A graph showing reusability (retained percentage activity) of laccase immobilized on various supports after 1–5 cycles, 5–10 cycles, 10–50 cycles and over 50 cycles.

8. Co-immobilization of laccase with other enzymes and substrates

Simultaneous immobilization of laccase with another or more enzymes has been explored to enhance the sensitivity and extend the substrate spectrum of biosensors [604, 605]. For example, Montereali *et al.* [606], reported a lower limit of detection and a wider range of phenolic compounds detected when a biosensor of co-immobilized laccase and tyrosinase on a ferrocene modified graphite screen printed electrode was used as compared to the individual laccase and tyrosinase biosensors. Moeder *et al.* [403], co-immobilized laccase and horse radish peroxidase (HRP) on a microporous polypropylene hollow fibre membrane by physical entrapment and the bioconjugate was used in the degradation of 4-ethylphenol, 3,4-dimethylphenol, tetralol, and 4-hydroxybiphenyl from water samples. Quan *et al.* [607], co-immobilized tyrosinase and laccase on a platinum electrode for use as a biosensor for PPD and *p*-chlorophenol. The sensitivity of the bi-enzyme sensor increased by 70% for PPD compared to only when the laccase sensor was used but the sensitivity of *p*-chlorophenol decreased by 40%. The increase in sensitivity towards PPD was due to additional catalytic function of the co-immobilized tyrosinase while the decrease in *p*-chlorophenol sensitivity was due to the blocking effect of the co-immobilized laccase which hinders mass transport through the immobilization layer. Similarly, ElKaoutit *et al.* [604], co-immobilized laccase and tyrosinase phenoxidase Sonogel–Carbon electrode using glutaric dialdehyde as crosslinker and nafion-ion exchanger as a protective additive. The electrode was used in a biosensing system to detect polyphenols in beer.

Co-immobilization of laccases from different sources has also been explored. For example, Vera *et al.* [608], co-immobilized laccase from *Aspergillus* sp., *M. thermophile*, and *T. versicolor* on poly (glycidyl methacrylate) microspheres. The biocatalyst showed a wide optimal pH range (2.0–7.0), high thermal, mechanical, and storage stabilities compared to the free and single-immobilized laccases.

9. Concluding remarks and future perspectives

Laccase has been widely studied due to its availability in fungi, plants, microorganisms, and insects. Since the enzyme is obtained from various sources, it demonstrates diversity in the composition of its carbohydrate moiety thus having varying secondary structures. This in turn affects the orientation of the enzyme active site hence different substrates are oxidized by different laccases from various sources. Nevertheless, laccase generally oxidizes a wide range of ortho and para substituted phenolic compounds using only molecular oxygen as the secondary substrate. This unique feature has allowed the study of the enzyme's use in various biotechnological applications including waste water treatment, dye decolourisation, paper and pulp industry, biofuel cells, pharmaceutical industry, and biosensors. However, most of the previous studies focused on the degradation of pollutants in aqueous solution which does not fully represent real industrial or environmental settings. Although the enzyme shows promising results in degradation of single organic pollutants, the complex physicochemical properties of the industrial effluents and surface water in the environment may not favour the smooth catalytic operation of the enzyme. The performance of the immobilized enzyme therefore needs to be evaluated in real wastewater, industrial effluents, and environmental conditions. More so, most of the studies have been done under batch conditions and yet continuous operations are preferred especially in industrial settings. It is therefore imperative to evaluate the performance of the biocatalyst under continuous operation while determining the optimal operational conditions for the process before industrial application can be realised.

Various supports ranging from organic to inorganic, porous to non-porous, and agricultural and industrial wastes have been used to immobilize laccase as discussed above. Since the performance of the biocatalyst depends on the type and structure of the enzyme, nature of the support, immobilization method and conditions, and intended use of the biocatalyst, consideration have to be made to maximize the catalytic and non-catalytic characteristics of the final biocatalyst.

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Declaration of interest's statement

The authors declare no competing interests.

Additional information

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