Laccases and their applications: A patent review

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Abstract: Laccases are an interesting group of multi copper enzymes, which have received much attention of researchers in last decades due to their ability to oxidize both phenolic and non-phenolic lignin related compounds as well as highly recalcitrant environmental pollutants. This makes these biocatalysts very useful for their application in several biotechnological processes. Such applications include the detoxification of industrial effluents, mostly from the paper and pulp, textile and petrochemical industries, polymer synthesis, bioremediation of contaminated soils, wine and beverage stabilization. Laccases are also used as catalysts for the manufacture of anti-cancer drugs and even as ingredients in cosmetics. Recently, the utility of laccases has also been applied to nanobiotechnology. This paper reviews recent and important patents related to the properties, heterologous production, molecular cloning, and applications of laccases within different industrial fields as well as their potential extension to the nanobiotechnology area.

Keywords: Laccases, Properties, Heterologous production, Molecular cloning, Industrial applications of enzymes, Food industry, Nanobiotechnology, Pulp and paper industry, Textile industry, Organic synthesis, Pharmaceutical sector, Bioremediation.

Running title: A patent review on laccases

INTRODUCTION

Laccase (EC 1.10.3.2) is a multicopper blue oxidase that couples the four-electron reduction of oxygen with the oxidation of a broad range of organic substrates, including phenols, polyphenols, anilines, and even certain inorganic compounds by a one-electron transfer mechanism [1-4]. Laccase is widely distributed in higher plants and fungi [5] and has been found also in insects and bacteria. Recently a novel polyphenol oxidase with laccase like activity was mined from a metagenome expression library from bovine rumen microflora [6]. Since their discovery more than one century ago in the Japanese tree *Rhus vernicifera* [7], laccases have been found to be widely distributed among plants, where they are involved in the synthesis of lignin and in the wounding response. Lignin, which provides the structural component of the plant cell wall, is a heterogeneous and complex biopolymer that consists of phenyl propanoid units linked by various non-hydrolyzable C-C and C-O bonds [8]. For many years, it was thought that only the ligninolytic system of some white-rot fungi capable of degrading this recalcitrant polymer to a major extent involved lignin peroxidase (LiP) and manganese peroxidase (MnP) [9]. Although the latter can only oxidize the phenolic components of lignin, lignin peroxidase -which has a high redox potential- is also capable of cleaving the non-phenolic aromatic part. The main limitation of all heme-containing peroxidases is their low operational stability, mostly due to their rapid deactivation by hydrogen peroxide. Also, the dependence of Mn^{2+} (for the MnP) or veratryl alcohol (for the LiP) are further shortcomings for their practical application. On the other hand, laccase alone is incapable of cleaving the non-phenolic bonds of lignin, and it was not considered a significant component of the ligninolytic system, despite the secretion of large quantities of laccase by these fungi under ligninolytic conditions. However, Bourbonnais and Paice [10] reported that laccases can catalyze the oxidation

of non-phenolic benzylalcohols in the presence of a redox mediator, such as 2,2'-azino-bis-[3-ethylthiazoline-6-sulfonate] (ABTS). This finding led to the discovery that laccase-mediator systems (LMS) effectively degrade residual lignin in unbleached pulp [11]. Indeed, laccases produced by some wood-rotting fungi from the genus *Basidiomycete* play a major role in the biodegradation of lignin [12] and have the capability to oxidize recalcitrant aromatic compounds with redox potentials exceeding their own with the help of natural or chemical mediators [2,13]. Because of their wide reaction capabilities as well as the broad substrate specificity, the laccase and the LMS possess great biotechnological potential. Promising applications include textile-dye bleaching [14], pulp bleaching [15], food improvement [16], bioremediation of soils and water [17,18], polymer synthesis [19], and the development of biosensors and biofuel cells [20,21].

The main aim of this work is to summarize the important patent literature data that has accumulated in the recent years about the properties, heterologous production and molecular cloning of laccases. In addition, applications of laccases within different industrial fields as well as their potential extension to the nanobiotechnology area, will also be discussed, particularly those appearing as published patents. Overall, this review is intended to discuss the laccases for biocatalysis and associated new patents.

PROPERTIES OF LACCASES

Current knowledge about the structure and physico-chemical properties of fungal proteins is based on the study of purified proteins. Up to now, more than 100 laccases have been purified from fungi and been more or less characterized. The laccase molecule, as an active holoenzyme form, is a dimeric or tetrameric glycoprotein, usually containing -per monomer- four copper (Cu) atoms bound to three redox sites (Type 1, Type 2 and Type 3 Cu pair). The molecular mass of the monomer ranges from about 50

to 100 kDa with acidic isoelectric point around pH 4.0. An important feature is the high level of glycosylation (with covalently linked carbohydrate moieties ranging from 10–50% of the total weight, depending on the species or the heterologous host), which may contribute to the high stability of the enzyme [22]. Several laccase isoenzymes have been detected in many fungal species. More than one isoenzyme is produced in most white-rot fungi.

Until recently, the three-dimensional structure of five fungal laccases has been reported: *Coprinus cinereus* (in a copper Type 2-depleted form) [23-26], *Trametes versicolor* [1,27], *Pycnoporus cinnabarinus* [28], *Melanocarpus albomyces* [29] and *Rigidoporus lignosus* [30], the latter four enzymes with a full complement of Cu ions. Moreover, the three-dimensional structure of the CoA laccase from *Bacillus subtilis* endospore has also recently been published [31,32].

For the catalytic activity a minimum of four Cu atoms per active protein unit is needed. Three types of copper can be distinguished using UV/visible and electronic paramagnetic resonance (EPR) spectroscopy. Type 1 Cu at its oxidised resting state is responsible for the blue colour of the protein at an absorbance of approximately 610 nm and is EPR detectable, Type 2 Cu does not confer colour but is EPR detectable and Type 3 Cu atoms consists of a pair of Cu atoms in a binuclear conformation that give a weak absorbance in the near UV region but no detectable EPR signal [33]. The Type 2 and Type 3 copper sites are close together and form a trinuclear centre that are involved in the catalytic mechanism of the enzyme [33].

The redox potential of the Type 1 site has been determined for many laccases using different mediators and varies from 430 mV for the laccase from *R. vernicifera* tree up to 780 mV for fungal laccase from *Polyporus versicolor* [3,24,34]. It was previously found that the catalytic efficiency (k_{cat}/K_m) of laccases for some reducing

substrates depended linearly on the redox potential of the Type 1 copper, in the sense that the higher the potential of the Type 1 site the higher the catalytic efficiency [3]. That is why laccases with a high redox potential of the Type 1 site are of special interest in biotechnology, e.g., for efficient bleaching and bioremediation processes.

Catalysis by laccase

To function, laccase depends on Cu atoms distributed among the three different binding sites. Cu atoms play an essential role in the catalytic mechanism. There are three major steps in laccase catalysis. The Type 1 Cu is reduced by a reducing substrate, which therefore is oxidized. The electron is then transferred internally from Type 1 Cu to a trinuclear cluster made up of the Type 2 and Type 3 Cu atoms (Fig. **1**). The O_2 molecule is reduced to water at the trinuclear cluster.

Place for Fig. **1**

The O_2 molecule binds to the trinuclear cluster for asymmetric activation and it is postulated that the O_2 binding pocket appears to restrict the access of oxidizing agents other than O_2. H_2O_2 is not detected outside of laccase during steady state laccase catalysis indicating that a four electron reduction of O_2 to water is occurring [34]. A one-electron substrate oxidation is coupled to a four-electron reduction of oxygen so the reaction mechanism cannot be straightforward. Laccase must operate as a battery, storing electrons from individual substrate oxidation reaction to reduce molecular oxygen. In fact, it appears that bound oxygen intermediates are also involved [34]. Details of the O_2 reduction have not been fully elucidated and continue to be studied.

From a mechanistic point of view, the reactions catalyzed by laccases for bioremediatory and biotechnological applications can be represented by one of the schemes shown in Fig. **2**. The simplest case (Fig. **2a**) is the one in which the substrate

molecules are oxidized to the corresponding radicals by direct interaction with the copper cluster. Laccases use oxygen as the electron acceptor to remove protons from the phenolic hydroxyl groups. This reaction gives rise to radicals that can spontaneously rearrange, which can lead to fission of C-C or C-O bonds of the alkyl side chains, or to cleavage of aromatic rings.

Frequently, however, the substrates of interest cannot be oxidized directly by laccases, either because they are too large to penetrate into the enzyme active site or because they have a particularly high redox potential. By mimicking nature, it is possible to overcome this limitation with the addition of so-called 'chemical mediators', which are suitable compounds that act as intermediate substrates for the laccase, whose oxidized radical forms are able to interact with the bulky or high redox-potential substrate targets (Fig. **2b**).

Place for Fig. **2**

Laccase mediator system (LMS)

Biobleaching techniques have been intensively investigated as a possible alternative for chlorine bleaching of pulp. It is known that white-rot fungi are able to perform lignin degradation using a cocktail of oxidative enzymes, including laccases, despite the fact that the bulkiness of this polymer prevents direct interaction with these enzymes. Indeed, it has been shown that the treatment of pulp with laccase alone does not catalyze the degradation of lignin but instead leads to only minor structural changes and repolymerization [35]. It has been hypothesized that small redox molecules might act as a sort of "electron shuttles" between the enzyme and the lignin and cause polymer de-branching and degradation [36]. Nowadays, this is regarded as more than just a hypothesis because the effect of chemical mediators, such as 3-hydroxyanthranilic acid

(HAA, Fig. **3a**), on laccase-catalyzed lignin degradation has been evaluated extensively [36]. The first artificial mediator that was used in the LMS for pulp delignification is ABTS (Fig. **3b**), which was introduced by Bourbonnais and Paice in 1990 [10]. Since then, over 100 possible mediator compounds have been tested for their ability to oxidize lignin or lignin models through the selective oxidation of their benzylic hydroxyl groups [37], and the most suitable ones are illustrated in Fig. **3**. The most effective mediators for lignin degradation proved to be the *N*-heterocycles bearing *N*–OH groups (Fig. **3c-g**) and in particular HBT (*N*-hydroxybenzotriazole; Fig. **3c**). The process has been patented under the trade name 'Lignozym® process', and its efficiency has been demonstrated in several pilot plant trials [37]. The evaluation of the performance of 12 different mediators in the oxidation of 4-methoxybenzyl alcohol, used as a model substrate, showed that TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl; Fig. **3h**) was the most effective compound for this kind of reaction [37]. A number of synthetic organic and inorganic mediators have been patented [36,38], and naturally occurring "native" mediators for laccases have been discovered and identified [39]. Moreover, the natural phenolic substrates of laccases, which are part of the extractive substances of wood, could also be the enhancers for the enzyme and increase the activity of laccase towards the lignin matrix during its destruction by fungi [40].

Place for Fig. **3**

The LMS concept was successfully applied to the oxidation of aromatic methyl groups, benzyl alcohols [41], polycyclic aromatic hydrocarbons (PAHs) [18,41] and bleaching of textile dyes [14]. However, despite that the addition of mediators may broaden the applicability of laccase, there are two major drawbacks hindering their use, they are expensive and are often toxic [41].

Various PAHs, which closely correlate to the 16 compounds selected by the Environmental Protection Agency (USA) and other national institutions as compounds of toxicological relevance were removed by a LMS. PAHs that were removed included acenaphtylene, anthracene, benzo(a)pyrene, acenaphthene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene and perylene [41]. PAH quinones were formed to differing degrees as oxidation products.

The activity of a LMS towards lignin is dependant on two main factors: firstly, the redox potential of the enzyme and, secondly, the stability and reactivity of the radical are resulting from the oxidation of the mediator. It has been shown that laccases from different organisms react variably with different mediators and different substrates [10]. It is thus imperative that different laccases as well as different mediator compounds be investigated.

Laccases of various origins differ in their substrate specificities and several substrates should be tested to assess a laccase activity. A very wide range of substrates has been shown to be oxidized by laccases (Table **1**) but the catalytic constants have been reported mostly for a small group of substrates- e.g. the non-natural test substrate ABTS and the phenolic compounds 2,6-dimethoxyphenol (2,6-DMP), guaiacol and syringaldazine. These constants have been measured for a large number of laccases, and rather great variation can be observed among them (Table **1**). The K_m values of laccases are in the range of 2-5000 μM, but differences as high as 3500-fold can be seen in the k_{cat} values between different laccases with the same substrates (Table **1**). The K_m values measured for 2,6-DMP are generally higher than those obtained with syringaldazine. The comparison of K_m values also shows that laccases from different source organisms have different substrate preferences [3]. Laccases in general combine high affinity for ABTS and syringaldazine with high catalytic constant, whereas the oxidation of

guaiacol and DMP is considerably slower and the respective K_m constants higher. On the other hand, the k_{cat} values for a single laccase do not generally differ more than 2-10-fold between different substrates, which reflects the fact that the k_{cat} describes the rate of the electron-transfer reactions taking place inside the enzyme after substrate binding [3]. This can be seen, for example, for laccases from *Pleurotus sajor-caju*, *Trametes pubescens* and *Trametes trogii* in Table **1**. However, the variance in assay conditions must always be taken into account when the catalytic constants measured in different laboratories are compared. The constants in Table 1 have been measured in varying pH, ionic strength and temperature conditions and using different protein concentrations, all of which have a great effect on the results. In addition, different molar extinction coefficients for oxidation products have sometimes been used in spectrophotometric assays, because the nature of the actual oxidation products is often complex or poorly understood. This affects particularly the numerical values of $k_{cat.}$

Place for Table **1**

Anions such as the halides, azide, cyanide and hydroxide bind to the Type 2 and 3 Cu atoms of laccases, which disrupts the electron transfer system, resulting in enzyme inhibition [34,42,43]. The inhibition by hydroxide generally prevents catalysis of substrates at alkaline pH [2]. The inhibition of activity by hydroxide prevents autoxidation at alkaline pH, with a resultant increase in stability at alkaline pH [2]. The inhibition by halides varies according to the laccase isozyme, and therefore likely related to the size of the channel of the trinuclear cluster (where oxygen binds) [2]. Other types of inhibitors include certain metal ions (e.g. Hg^{2+}), fatty acids, sulfhydril reagents, hydroxyglycine, kojic acid, and cationic quaternary ammonium detergents

[34]. These compounds may affect the laccase by chelating the Cu (II) atoms, by modifying of amino acid residues or they may elicit a conformational change in the glycoprotein.

HETEROLOGOUS PRODUCTION OF LACCASES

In most fungi, laccases are produced in the native hosts at levels that are too low for commercial purposes. Therefore, improving the productivity and reducing the production cost are the major goals for the current studies on laccase production. Cloning of the laccase genes followed by heterologous expression may provide higher enzyme yields.

In order to improve laccase production, fungal laccases have been expressed heterologously in *Saccharomyces cerevisiae* [48,49,60-63], *Trichoderma reesei* [48,54,64], *Aspergillus oryzae* [65-71], *Pichia pastoris* [51,72-76], *Yarrowia lipolitica* [77], *Aspergillus sojae* [78], *Aspergillus niger* [52,79], *Aspergillus nidulans* [69,79,80], *Aspergillus ficuum* [81], tobacco [82-85] and maize [86-88]. Bacterial laccases from *B. subtilis, Thermus thermophilus* and *Streptomyces lavendulae* have been expressed in *Escherichia coli* [44,89-91] but successful expression of fungal laccases in *E. coli* has not been reported.

In spite of the fact that laccase production levels have often been improved significantly by expression in heterologous hosts, the reported levels are still rather low for industrial applications (Table **2**). Improved laccase production levels have also been achieved by expression in *P. pastoris*, whereas expression in *S. cerevisiae* has generally resulted in very low activity levels [92]. Recently, successful use of *S. cerevisiae* expressing *M. thermophila* laccase by directed evolution was reported by Bulter et al., who obtained very promising results [49]. The addition of copper into the culture medium also proved to be important for heterologous laccase

production in *P. pastoris* and *Aspergillus* spp. [75,79,93]. The importance of adequate copper concentration for proper laccase folding was further corroborated by studies in which two genes related to copper-trafficking in *T. versicolor* were overexpressed in *S. cerevisiae* expressing *T. versicolor lacIII* gene: the heterologous laccase production by *S. cerevisiae* was improved up to 20-fold [94,95]. The effect was suggested to result from more efficient transport of copper to the Golgi compartment [94,95]. Also, the improved laccase production levels have been obtained in heterologous production systems in a laboratory-scale fermentor cultivation by expression of *M. albomyces* in *T. reesei*, which yielded 920 mg laccase per liter [63]. Recently, an efficient transformation and expression system was developed for the basidiomycete *P. cinnabarinus* and this was used to transform a laccase-deficient monokaryotic strain with the homologous *lac1* laccase gene. The yield obtained was as high as 1200 mg l^{-1} and represents the highest laccase production reported for recombinant fungal strains [96,97]. It has not been established whether production levels in a strain that already produces such high levels of laccase can be further increased by genetic modification.

Place for Table **2**

cDNA AND GENE SEQUENCES

The first gene and/or cDNA sequences were recorded for laccase from the Ascomycete fungus, *Neurospora crassa* [98], and sequences were published from 1990 onwards. These included laccases from *A. nidulans* [99], *Coriolus hirsutus* [60,62], *Phlebia radiata* [100], *Agaricus bisporus* [101], *P. cinnabarinus* [102], *Coriolus versicolor* [103], *T. versicolor* [72], *Podospora anserina* [104], *Coprinus congregatus* [105], *Ganoderma lucidum, Phlebia brevispora, Lentinula edodes* and *Lentinus tigrinus*

[42,106]. Since then, the number of laccase genes sequenced has increased considerably, and searches from protein and gene sequence databases currently yield several hundreds of laccase gene sequences. However, a significant number of these are only partial stretches of putative laccase genes that have been found in genome-wide sequencing projects and have been annotated on the basis of sequence homology with known laccases. The number of laccase genes of which the corresponding protein products have been experimentally characterized is significantly lower. To date, there are about 21 such enzymes, most of which are fungal laccases (Table **3**). In addition to the genes shown in Table **3**, several laccase genes have been characterized in detail at the nucleotide level but have not been specified to code for a known laccase protein.

The sequences mostly encode polypeptides of approximately 500 to 600 amino acids (including the N-terminal secretion peptide) (Table **3**). All the laccases listed in Table 3 are secreted proteins, and typical eukaryotic signal peptide sequences of about 21 amino acids are found at the N-termini of the protein sequences. In addition to the secretion signal sequence, laccase genes from *N. crassa*, *P. anserina*, *M. thermophila* and *C. cinereus* contain regions that code for N-terminal cleavable propeptides [66,69,104,107]. These laccases also have C-terminal extensions of controversial function, i.e. the last amino acids from the predicted amino acid sequence are not present in the mature protein [66,69,107].

The one cysteine and ten histidine residues involved in the binding of copper atoms were conserved for laccases and this is also similar to what is found for sequences from ascorbate oxidase. The difference between laccases and ascorbate oxidases in the copper-binding region is that the latter exhibits the presence of a methionine axial ligand, which is not present in the laccase sequences. The

absence/presence of the methionine ligand has led to interesting studies of mutagenesis conducted by Xu and coworkers [108,109].

Place for Table **3**

APPLICATIONS OF LACCASES

A few laccases are at present in market for textile, food and other industries, and more candidates are being actively developed for future commercialization. A vast amount of industrial applications for laccases have been proposed and they include pulp and paper, textile, organic synthesis, environmental, food, pharmaceuticals and nanobiotechnology. Being specific, energy-saving, and biodegradable, laccase-based biocatalysts fit well with the development of highly efficient, sustainable, and eco-friendly industries.

PULP AND PAPER INDUSTRY

In the industrial preparation of paper the separation and degradation of lignin in wood pulp are conventionally obtained using chlorine- or oxygen-based chemical oxidants. Non-chlorine bleaching of pulp with laccase was first patented in 1994 using an enzyme treatment to obtain a brighter pulp with low lignin content [119]. Oxygen delignification process has been industrially introduced in the last years to replace conventional and polluting chlorine-based methods. In spite of this new method, the pre-treatments of wood pulp with laccase can provide milder and cleaner strategies of delignification that also respect the integrity of cellulose [120-123]. Laccases are able to delignify pulp when they are used together with mediators [124]. Small natural low-molecular weight compounds with high redox potential (>900 mV) called mediators may be used to oxidize the non-phenolic residues from the oxygen delignification [124].

The mediator is oxidized by laccase and the oxidized mediator molecule further oxidizes subunits of lignin that otherwise would not be laccase substrates [10,125]. Although the LMS has been studied extensively, there are still unresolved problems concerning with mediator recycling, cost and toxicity. However, some environmental benefits are envisaged and the fact that LMS could be easily implemented in the existing bleaching sequences is seen as a major advantage that could possibly lead to a partial replacement of ClO_2 in pulp mills. Furthermore, the application of laccases in pulp-kraft bleaching may result in higher pulp yields and energy savings. Most of studies have been patented about the use of LMS in the pulp-kraft bleaching processes [123,126-141]. More recently, the potential of this enzyme for cross-linking and functionalizing ligninaceous compounds was discovered [142-145]. In another related application, laccases can be even used for deinking and decolorizing a printed paper [146-149]. Finally, laccases can be used for binding fiber-, particle- and paper-boards [143,150-153]. However, different wood-decaying basidiomycetes have shown a highly variable pattern of laccase formation, and this subject requires more detailed experiments [154].

TEXTILE INDUSTRY

Laccase is used in commercial textile applications to improve the whiteness in conventional bleaching of cotton and recently biostoning [155-157]. Potential benefits of the application include chemicals, energy, and water saving. Cellulases were used to partially replace the load of pumice stones and laccases could bleach indigo-dyed denim fabrics to lighter shades [158-163]. Laccase also can be used *in situ* to convert dye precursors for better, more efficient fabric dyeing [160,164-166]. In the last few years, various patents reported on coloration achieved with laccase [164,167-173].

Laccases find potential applications for cleansing, such as cloth- and dishwashing [174-176]. Laccase may be included in a cleansing formulation to eliminate the odor on fabrics, including cloth, sofa surface, and curtain, or in a detergent to eliminate the odor generated during cloth washing [70,177-179]. A patent application about the use of LMS to increase the shrink resistance of wool was published [180]. Also, Lantto et al. [181] found that wool fibers can be activated with LMS. Therefore, the use of laccase for anti-shrink treatment of wool seems very attractive.

FOOD INDUSTRY

Many laccase substrates, such as carbohydrates, unsaturated fatty acids, phenols, and thiol-containing proteins, are important components of various foods and beverages. Their modification by laccase may lead to new functionality, quality improvement, or cost reduction [16,174]. Sometimes O_2 is detrimental to the quality or storage of food/beverage because of unwanted oxidation. Laccases may be used as O_2-scavengers for better food packing [182].

The flavor quality of vegetable oils can be improved with laccase by eliminating dissolved oxygen [183]. Laccase can also deoxygenate food items derived partly or entirely from extracts of plant materials. Cacao was soaked in solutions containing laccase, dried and roasted in order to improve the flavor and taste of cacao and its products [184,185]. The reduction of odors with laccase is documented in the patent literature [186,187]. Treatment with a fungal laccase can also be performed to enhance the color of a tea-based product [81,188-190]. It is also used to perform the cross-link of ferulic acid and sugar beet pectin through oxidative coupling to form gels for food ingredients [60,191]. Various enzymatic treatments have been proposed for fruit juice stabilization, among which it can be found the use of laccase [16,192,193]. Laccases are added to the dough used for producing baked products, to exert an oxidizing effect on

the dough constituents and to improve the strength of gluten structures in dough and/or baked products [16,81,194].

Wine stabilization is one of the main applications of laccase in the food industry as alternative to physical-chemical adsorbents [16,195-198]. Musts and wines are complex mixtures of different chemical compounds, such as ethanol, organic acids (aroma), salts and phenolic compounds (color and taste). Polyphenol removal must be selective to avoid an undesirable alteration in the wine's organoleptic characteristics. Laccase presents some important requirements when used for the treatment of polyphenol elimination in wines, such as stability in acid medium and reversible inhibition with sulphite [199]. Laccases are also used to improve storage life of beer. Haze formation in beers is a persistent problem in the brewing industry. Nucleophilic substitution of phenolic rings by protein sulphydryl groups may lead to a permanent haze that does not re-dissolve when warmed. As an alternative to the traditional treatment to remove the excess of polyphenols, laccase could be added to the wort [16,200]. Other studies of laccase application for phenolic compounds removal have also been patented [185,201]. A laccase has recently been commercialized (Suberzyme®) for preparing cork stoppers for wine bottles [202].

BIOREMEDIATION

Laccases have many possible applications in bioremediation [159]. Laccases may be applied to degrade various substances such as undesirable contaminants, by-products, or discarded materials. Laccase may be applied to degrade plastic waste having olefin units [203,204]. Likely, an oxidation of the olefin units by the LMS, could initiate a radical chain reaction, leading to the disintegration of the plastic. Also this LMS can be used to degrade polyurethanes [205]. LMS facilitated the degradation of phenolic compounds (environmental hormones) from biphenol and alkylphenol

derivatives [206,207] and also the decomposition of fluorescent brighteners [208,209]. Laccase may also be used to eliminate odor emitted from places such as garbage disposal sites, livestock farms, or pulp mills [177,210]. Also, they could be used for decolorizing dye house effluents that are hardly decolorized by conventional sewage treatment plants [42,211-213]. In addition to dye house effluents, laccases can decolorize waste waters from olive oil mills [214] and pulp mills [215,216] by removing colored phenolic compounds.

Another potential environmental application for laccases is the bioremediation of contaminated soils, as laccases and LMS are able to oxidize toxic organic pollutants, such as various xenobiotics, PAHs, chlorophenols, and other contaminants [16,89,130,177,210,218-226]. Phenolic compounds are present in wastes from several industrial processes, as coal conversion, petroleum refining, production of organic chemicals and olive oil production among others [227,228]. Immobilized laccase was found to be useful to remove phenolic and chlorinated phenolic pollutants [129,229-233]. Laccase was found to be responsible for the transformation of 2,4,6-trichlorophenol to 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone [88,234]. LMSs have been also used to oxidize alkenes, carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene [235,236]. Isoxaflutole is an herbicide activated in soils and plants to its diketonitrile derivative, the active form of the herbicide: laccases are able to convert the diketonitrile into the acid [237]. Laccase can be also used to reduce the concentration of synthetic heterocyclic compound such as halogenated organic pesticides in the soil [218]. LMS has been extensively study in the oxidation of recalcitrant PAHs, main components of several ship spills. In this sense, LMS is being included in several enzymatic bioremediation programs [238].

ORGANIC SYNTHESIS

Recently, increasing interest has been focused on the application of laccase as a new biocatalyst in organic synthesis [154,239]. Laccase provided an environmentally benign process of polymer production in air without the use of H_2O_2 [240,241]. Laccase-catalyzed cross-linking reaction of new urushiol analogues for the preparation of "artificial urushi" polymeric films (Japanese traditional coating) was demonstrated [242]. It is also mentioned that laccase induced radical polymerization of acrylamide with or without mediator [243,244]. Laccases are also known to polymerize various amino and phenolic compounds [245-248]. Recently, to improve the production of fuel ethanol from renewable raw materials, laccase from *T. versicolor* was expressed in *S. cerevisiae* to increase its resistance to (phenolic) fermentation inhibitors in lignocellulose hydrolyzates [92]. The preparation of crosslinked enzyme aggregrates with aldehydes and amines had improved stability and was used in starch oxidation [249]. Selective oxidation of the primary hydroxyl groups of sugars using the LMS is described in patent literature dealing with the partial oxidation of cellulose and other polysaccharides [250-254]. Also, LMS was used for the determination of monoclonal antibody of azelaic acid from oleic acid [255]. The enzymatic preparation of polymeric polyphenols by the action of laccases has been investigated extensively in the past decades as a viable and non-toxic alternative to the usual formaldehyde-based chemical production of these compounds [256-260].

Laccase can also be used to synthesize various functional organic compounds including polymers with specific mechanical/electrical/optical properties, textile dyes, cosmetic pigments, flavor agents, and pesticides [110,261-263].

PHARMACEUTICAL SECTOR

Many products generated by laccases are antimicrobial, detoxifying, or active personal-care agents. Due to their specificity and bio-based nature, potential applications of laccases in the field are attracting active research efforts. Laccase can be used in the synthesis of complex medical compounds as anesthetics, anti-inflammatory, antibiotics, sedatives, etc. [264-267], including triazolo(benzo)cycloalkyl thiadiazines, vinblastine, mitomycin, penicillin X dimer, cephalosporins, and dimerized vindoline [159,268,269].

One potential application is laccase-based *in situ* generation of iodine, a reagent widely used as disinfectant [159,270,271]. Also, laccase has been reported to possess significant HIV-1 reverse transcriptase inhibitory activity [272]. Another laccase has been shown capable of fighting aceruloplasminemia (a medical condition of lacking ceruloplasmin, a multi-Cu serum oxidase whose ferroxidase activity regulates iron homeostasis) [273]. Some years ago, a new enzymatic method based on laccase was developed to distinguish simultaneously morphine and codeine in drug samples injected into a flow detection system [274].

A novel application field for laccases is in cosmetics. For example, laccase-based hair dyes could be less irritant and easier to handle than current hair dyes [159,275-289]. More recently, cosmetic and dermatological preparations containing proteins for skin lightening have also been developed [261,288,290-292]. Laccases may find use as deodorants for personal-hygiene products, including toothpaste, mouthwash, detergent, soap, and diapers [177,293-300]. Protein engineered laccase may be used to reduce allergenicity [301].

NANOBIOTECHNOLOGY

Nanoscience has grown rapidly in the last decade. Recently, more attention is focused on the applications of nanotechnologies. The high potential impacts of nanotechnology almost cover all fields of human activity (environmental, economy, industrial, clinical, health-related, etc). Nanostructured materials (nanoparticles, nanotubes, and nanofibers) have been used extensively as carrying materials for biosensoring, and biofuel cells.

A biosensor is an integrated biological-component probe with an electronic transducer, thereby converting a biochemical signal into a quantifiable electrical response that detects, transmits and records information regarding a physiological or biochemical change [302]. Some of the major attributes of a good biosensing system are its specificity, sensitivity, reliability, portability, real-time analysis and operation simplicity [302]. Thus laccases can be applied as biosensors or bioreporters. A number of biosensors containing laccase have been developed for immunoassays, and for determination of glucose, aromatic amines and phenolic compounds [159,274,303-308]. Laccase catalysis can be used to assay other enzymes [159,309-311]. Laccase covalently conjugated to a bio-binding molecule can be used as a reporter for immunochemical (ELISA, Western blotting), histochemical, cytochemical, or nucleic acid-detection assays [80,159,312,313]. The bioreporter applications are of interest for the high-sensitivity diagnostic field.

In addition to biosensors, laccases could be immobilized on the cathode of biofuel cells that could provide power, for example, for small transmitter systems [314-317]. Fuel cells are very attractive energy sources, particularly at micro-, mini-, portable-, or mobile-scale, that potentially have higher energy conversion/usage efficiency and lower pollution effect than any of the existing/emerging energy sources.

For example, a bio-implantable electrochemical cell system for active implantable medical devices is described by Choi [318]. In one embodiment, the fuel cell includes an electrode structure consisting of immobilized anode and cathode enzymes deposited on nanostructured high-surface-area metal nanowires or carbon nanotube electrodes (Fig. **4**). The anode enzyme comprises immobilized glucose oxidase and the cathode enzyme comprises immobilized laccase. Glucose is oxidized at the surface of the anode and oxygen is reduced at the surface of the cathode. The coupled glucose oxidation/oxygen reduction reactions provide a self-generating current source. In another embodiment, the nanowires or carbon nanotubes, along with the adjacent surface anode and cathode electrodes, are coated with immobilized glucose oxidase and immobilized laccase containing biocolloidal substrates, respectively. This results in the precise construction of enzyme architecture with control at the molecular level, while increasing the reactive surface area and corresponding output power by at least two orders of magnitude.

Place for Fig. **4**

Laccase may be applied as a biocatalyst for the electrode reactions [319-324]. Laccase-based miniature biological fuel cell is of particular interest for many medical applications calling for a power source implanted in a human body [325].

POTENTIAL NEW LACCASE-BASED BIOCATALYSTS

Enzymatic catalysis in organic solvents has opened a new field of biotechnological applications of enzymes. The ability to use enzymes in nonaqueous solvents greatly expands the potential scope and economic impact of biocatalysis. When biological catalysts are placed in this unnatural environment they exhibit a number of remarkable novel properties such as altered stereo-selectivity, enhanced stability and

increased rigidity [326]. There is also facilitated recovery of products and biocatalyst [327]. As well in the presence of organic solvents there is less risk of microbial contamination [328]. In the specific case of laccases or LMS, many of their non-natural substrate are hardly soluble in water (their K*m* values are far away from their solubility in aqueous media), and therefore the use of cosolvents is an indispensable requirement. However, the laccase may be denatured or it may be inhibited under these conditions [329,330]. In this regard laccases can be applied in nonaqueous solution or multiphasic systems. For water-immiscible organic solvents, laccase may be entrapped in reverse micelles or immobilized onto a carrier [22,159,232,331]. Preferably, the reverse micelles are used in the presence of laccase mediators to enhance and mediate the laccase activity in organic solvents. Solid phase enzyme kinetics screening in micro-colonies provide higher throughput, better solvent resistance and easy of handling [332]. In a recent investigation directed molecular evolution of laccases was carried out for organic cosolvents resistance [333]. The *M. thermophila* laccase expressed in *S. cerevisiae* was engineered by *in vitro* evolution in the presence of increasing concentrations of acetonitrile and ethanol. The turnover rates of mutant enzymes at high concentrations of organic solvents were several fold improved (our laboratory has already perfomed new cycles of *in vitro* evolution, achieving high activities at solvent concentrations as high as 50 % (v/v) (unpublished material).

CURRENT AND FUTURE DEVELOPMENTS

This review summarizes the available recent and important patents about the properties, heterologous production, and molecular cloning of fungal laccases and possible industrial and biotechnological use.

Laccases are promising enzymes to replace the conventional chemical processes of several industries such as the pulp and paper, textile, pharmaceutical, and

nanobiotechnology. However, one of the problems to commercialise the use of laccase is the lack of sufficient enzyme stocks. Thus, efforts have to be made in order to achieve cheap overproduction of laccase in heterologous hosts, and also their modification by chemical means or protein engineering, to obtain more robust, active and less expensive enzymes. Another additional problem is the cost and toxicity of redox mediators. Further investigations should consider different and less polluting mediators such as the natural mediators produced by laccase in a bio-environment during lignin degradation [13]. The current development in laccase catalysis research and the design of mediators along with the research on its heterologous expression opens a wide spectrum of possible applications in the near future. Moreover, laccase can also offer a simple and convenient alternative to using peroxidases with H_2O_2, because laccases are available on an economically feasible scale.

ABBREVIATIONS

LiP = Lignin peroxidase

MnP = Manganese peroxidase

ABTS = 2,2'-azino-bis-[3-ethylthiazoline-6-sulfonate]

LMS = Laccase-mediator system

Cu = Copper

EPR = Electronic paramagnetic resonance

HAA = 3-hydroxyanthranilic acid

HBT = *N*-hydroxybenzotriazole

TEMPO = 2,2,6,6-tetramethylpiperidine-1-oxyl

2,6-DMP = 2,6-Dimethoxyphenol

NHA = *N*-hydroxyacetanilide

HPI = *N*-hydroxyphtaimide

VLA = Violuric acid

PAH = Polycyclic aromatic hydrocarbon

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Table 1. Kinetic constants of laccases. The pH-values at which the constants have been measured are also included.

Substrate	K_m (μM)	k_{cat} (min^{-1})	pH	Laccase	Reference
ABTS	106	1000	4.0	*Bacillus subtilis* CotA	Martins et al. [44]
	190	n.r.*	6.0	*Chaetomium thermophilum*	Chefetz et al. [45]
	23	1090	5.5	*Coprinus cinereus* Lcc1	Schneider et al. [24,46]
	41	n.r.	5.0	*Coprinus friesii*	Heinzkill et al. [47]
	270	4690	4.5	*Melanocarpus albomyces*	Kruus et al. [48]
	290	790	6.0	*Myceliophthora thermophila* Lcc1	Bulter et al. [49]
	32	n.r.	3.0	*Panaeolus sphinctrinus*	Heinzkill et al. [47]
	50.6	n.r.	3.0	*Panaeolus papilionaceus*	Heinzkill et al. [47]
	90	350000	3.0	*Pleurotus ostreatus* POXA1	Palmieri et al. [50]
	120	n.r.	3.0	*Pleurotus ostreatus* POXA2	Palmieri et al. [50]
	280	57000	3.0	*Pleurotus ostreatus* POXC	Palmieri et al. [50]
	2500	74000	3.3	*Pleurotus sajor-caju* Lac4	Soden et al. [51]
	55	n.r.	4.0	*Pycnoporus cinnabarinus* Lac1	Record et al. [52]
	52	n.r.	5.3	*Rhizoctonia solani* Lcc4	Xu et al. [3]
	380	n.r.	4.5	*Streptomyces cyaneus*	Arias et al. [53]
	75	4130	4.5	*Thielavia arenaria Lcc1*	Paloheimo et al. [54]
	14	41400	3.0	*Trametes pubescens* LAP2	Galhaup et al. [55]
	30	198	3.4	*Trametes trogii* POXL3	Garzillo et al. [56]
	58	2700	5.3	*Trametes villosa* Lcc1	Xu et al. [3]
	45	620	5.5	*Trichophyton rubrum*	Jung et al. [57]
2.6-DMP	100	n.r.	3.5	*Botrytis cinerea*	Slomczynski et al. [58]
	96	n.r.	6.0	*Chaetomium thermophilum*	Chefetz et al. [45]
	26	n.r.	4.5	*Gaeumannomyces graminis* LAC2	Edens et al. [59]
	5	4160	6.0	*Melanocarpus albomyces*	Kruus et al. [48]
	2100	21000	5.0	*Pleurotus ostreatus* POXA1	Palmieri et al. [50]
	740	n.r.	6.5	*Pleurotus ostreatus* POXA2	Palmieri et al. [50]
	230	430	5.0	*Pleurotus ostreatus* POXC	Palmieri et al. [50]
	120	58000	6.0	*Pleurotus sajor-caju* Lac4	Soden et al. [51]
	17	4030	6.0	*Thielavia arenaria Lcc1*	Paloheimo et al. [54]
	72	24000	3.0	*Trametes pubescens* LAP2	Galhaup et al. [55]
	410	109	3.4	*Trametes trogii* POXL3	Garzillo et al. [56]
Guaiacol	400	n.r.	6.0	*Chaetomium thermophilum*	Chefetz et al. [45]
	510	n.r.	4.5	*Gaeumannomyces graminis* LAC2	Edens et al. [59]
	3100	n.r.	6.0	*Pleurotus ostreatus* POXA2	Palmieri et al. [50]

	1200	150	6.0	*Pleurotus ostreatus* POXC	Palmieri et al. [50]
	66	6800	6.5	*Pleurotus sajor-caju* Lac4	Soden et al. [51]
	36	10800	3.0	*Trametes pubescens* LAP2	Galhaup et al. [55]
	5120	115	3.4	*Trametes trogii* POXL3	Garzillo et al. [56]
Syringaldazine	26	200	6.0	*Bacillus subtilis* CotA	Martins et al. [44]
	34	n.r.	6.0	*Chaetomium Thermophilum*	Chefetz et al. [45]
	26	180	5.5	*Coprinus cinereus* Lcc1	Schneider et al. [24,46]
	1.3	4710	6.0	*Melanocarpus albomyces*	Kruus et al. [48]
	1.6	2100	6.0	*Myceliophthora thermophila* Lcc1	Bulter et al. [49]
	130	28000	6.0	*Pleurotus ostreatus* POXA1	Palmieri et al. [50]
	140	n.r.	6.0	*Pleurotus ostreatus* POXA2	Palmieri et al. [50]
	20	23000	6.0	*Pleurotus ostreatus* POXC	Palmieri et al. [50]
	280	35000	6.5	*Pleurotus sajor-caju* Lac4	Soden et al. [51]
	28	n.r.	5.3	*Rhizoctonia solani* Lcc4	Xu et al. [3]
	4.3	1940	6.0	*Thielavia arenaria Lcc1*	Paloheimo et al. [54]
	6	16800	4.5	*Trametes pubescens* LAP2	Galhaup et al. [55]
	3.9	3000	5.3	*Trametes villosa* Lcc1	Xu et al. [3]

*n.r., not reported

Table 2. Laccase production in heterologous hosts.

Laccase gene	Production host	Laccase Production (mg l^{-1})*	Reference
Ceriporiopsis subvermispora lcs-1	*Aspergillus nidulans*	1.5	Larrondo et al. [79]
	Aspergillus niger	1.5	Larrondo et al. [79]
Coprinus cinereus lcc1	*Aspergillus oryzae*	135	Yaver et al. [66]
Melanocarpus albomyces lac1	*Trichoderma reesei*	920	Kiiskinen et al. [63]
Myceliophthora thermophila lcc1	*Aspergillus oryzae*	19	Berka et al. [68,69]
	Saccharomyces cerevisiae	18	Bulter et al. [49]
Phlebia radiata lac1	*Trichoderma reesei*	20	Saloheimo and Niku-Paavola [64]
Pleurotus sajor-caju lac4	*Pichia pastoris*	4.9	Soden et al. [51]
Pycnoporus cinnabarinus lac1	*Pichia pastoris*	8	Otterbein et al. [73]
	Aspergillus niger	70	Record et al. [52]
	Aspergillus oryzae	80	Sigoillot et al. [71]
	Schizophyllum commune	1200	Alves et al. [96,97]

* The reported production levels have been obtained in shake flask cultivation, except in the case of *P. radiata* and *M. albomyces* laccases which were produced in a laboratory fermentor.

Table 3. Examples of laccase genes that have been shown to encode a biochemically characterized laccase protein.

Organism	Gene		Protein encoded by the gene	Reference
	Name	**EMBL Acc.No.**	**Length (aa)**	
Bacillus subtilis	*cotA*	U51115	513	Martins et al. [44]
Botrytis cinerea	*Bclcc2*	AF243855	581	Schouten et al. [110]
Ceriporiopsis subvermispora	*lcs-1*	AY219235	519	Karahanian et al. [111]
Coprinus cinereus	*lcc1*	AF118267	539	Yaver et al. [66]; Schneider et al. [24]
Cryptococcus neoformans	*CNLAC1*	L22866	624	Williamson [112]
Gaeumannomyces graminis var. *tritici*	*LAC2*	AJ417686	577	Edens et al. [59]
Marasmius quercophilus (Basidiomycete C30)	*lac1*	AF162785	517	Dedeyan et al. [113]
Melanocarpus albomyces	*Lac 1*	AJ57169	623	Kruus et al. [48]
Myceliophthora thermophila	*lcc1*	AR023901	619	Berka et al. [68,69]
Neurospora crassa	*2 alleles*	M18333-4	619	Germann et al. [107]
Phlebia radiata	*lac1*	X52134	548	Saloheimo et al. [100]
Pleurotus ostreatus	*poxa1b*	AJ005017	533	Giardina et al. [114]
Pleurotus ostreatus	*poxc (=pox2)*	Z49075	533	Giardina et al. [115]
Basidiomycete PM1 (CECT 2971)	*lac1*	Z12156	517	Coll et al. [12]
Podospora anserina	*lac2*	Y08827	621	Fernandez-Larrea and Stahl [104]
Populus euramericana	*lac90*	Y13772	574	Ranocha et al. [116]
Rhizoctonia solani	*lcc4*	Z54277	530	Wahleithner et al. [67]
Streptomyces lavendulae	-	AB092576	631	Suzuki et al. [89]
Trametes pubescens	*lap2*	AF414807	523	Galhaup et al. [55]
Trametes trogii	*lcc1*	Y18012	496	Garzillo et al. [56]
Trametes versicolor	*lcc1*	L49376	519	Bourbonnais et al. [117]

Trametes versicolor	*lcc2*	U44430	520	Cassland and Jonsson [118]
Trametes villosa	*lcc1*	L49377	520	Yaver et al. [65]
Trametes villosa	*lcc2*	AY249052	519	Yaver et al. [65]

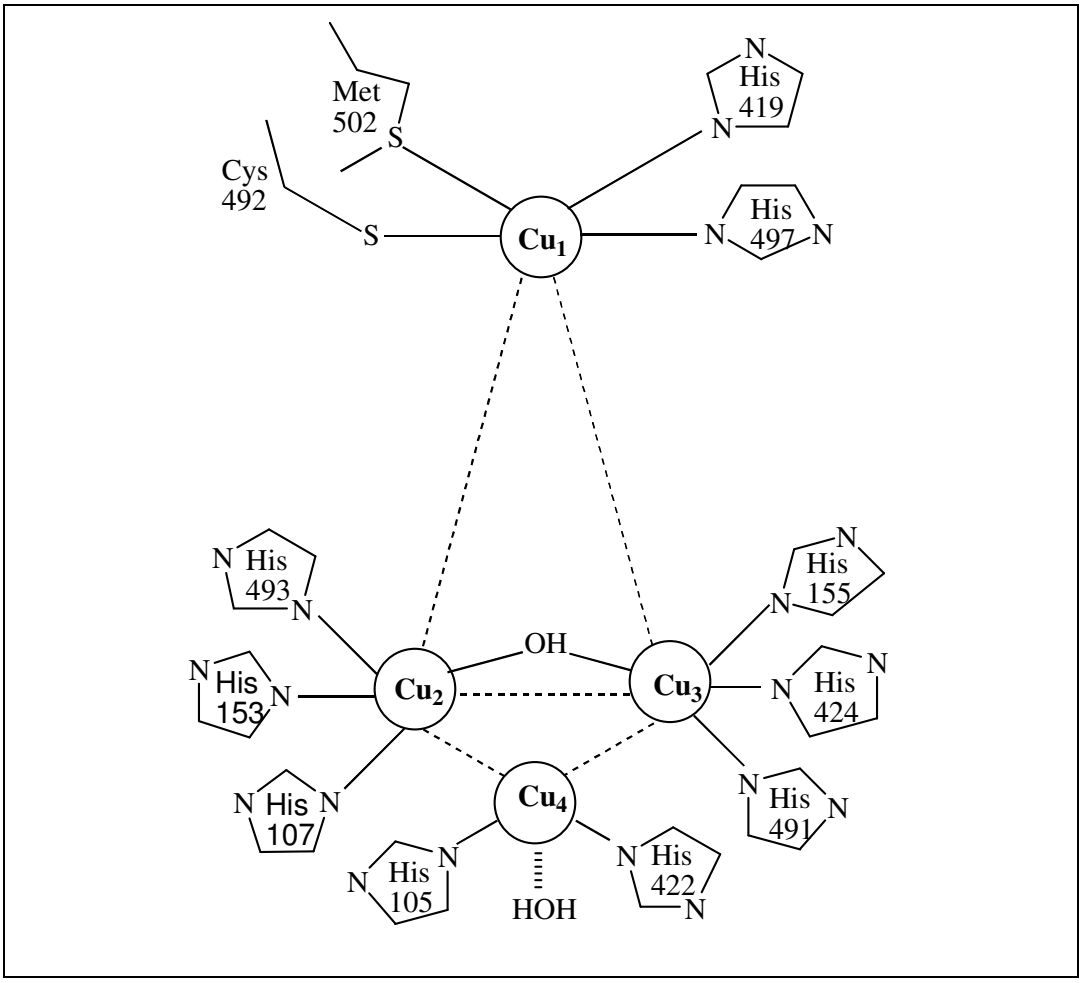

Fig. 1. Illustration of the active site of laccase showing the relative orientation of the copper atoms [31].

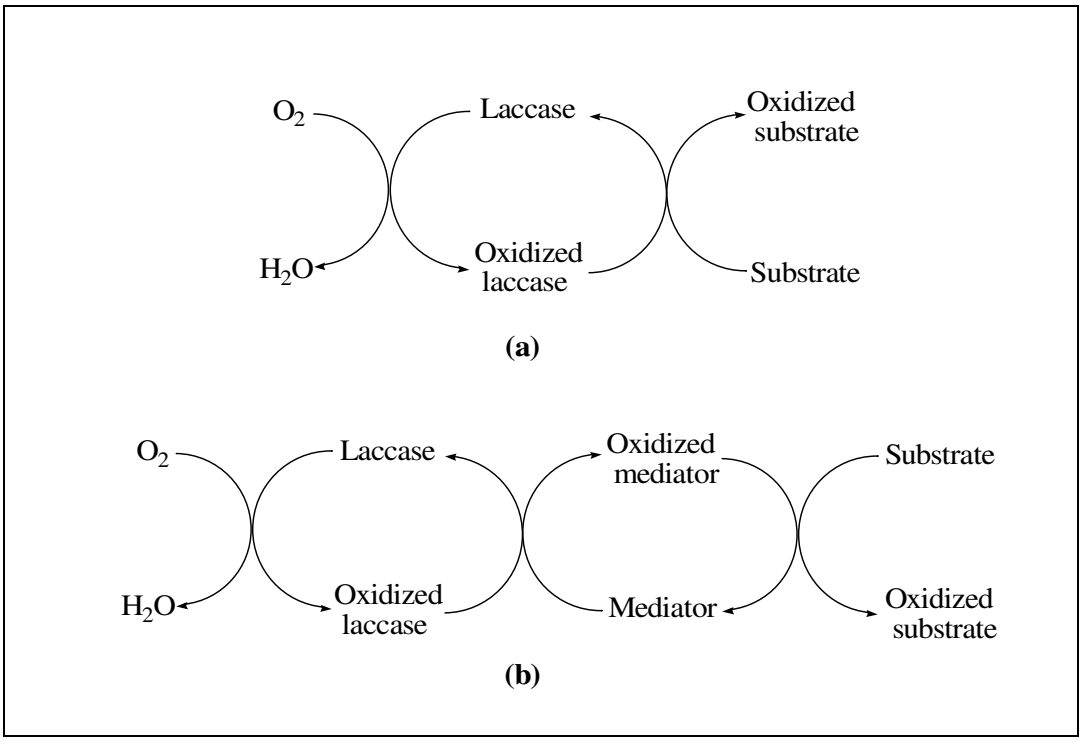

Fig. 2. Schematic representation of laccase-catalyzed redox cycles for substrates oxidation in the absence **(a)** or presence **(b)** of chemical mediators.

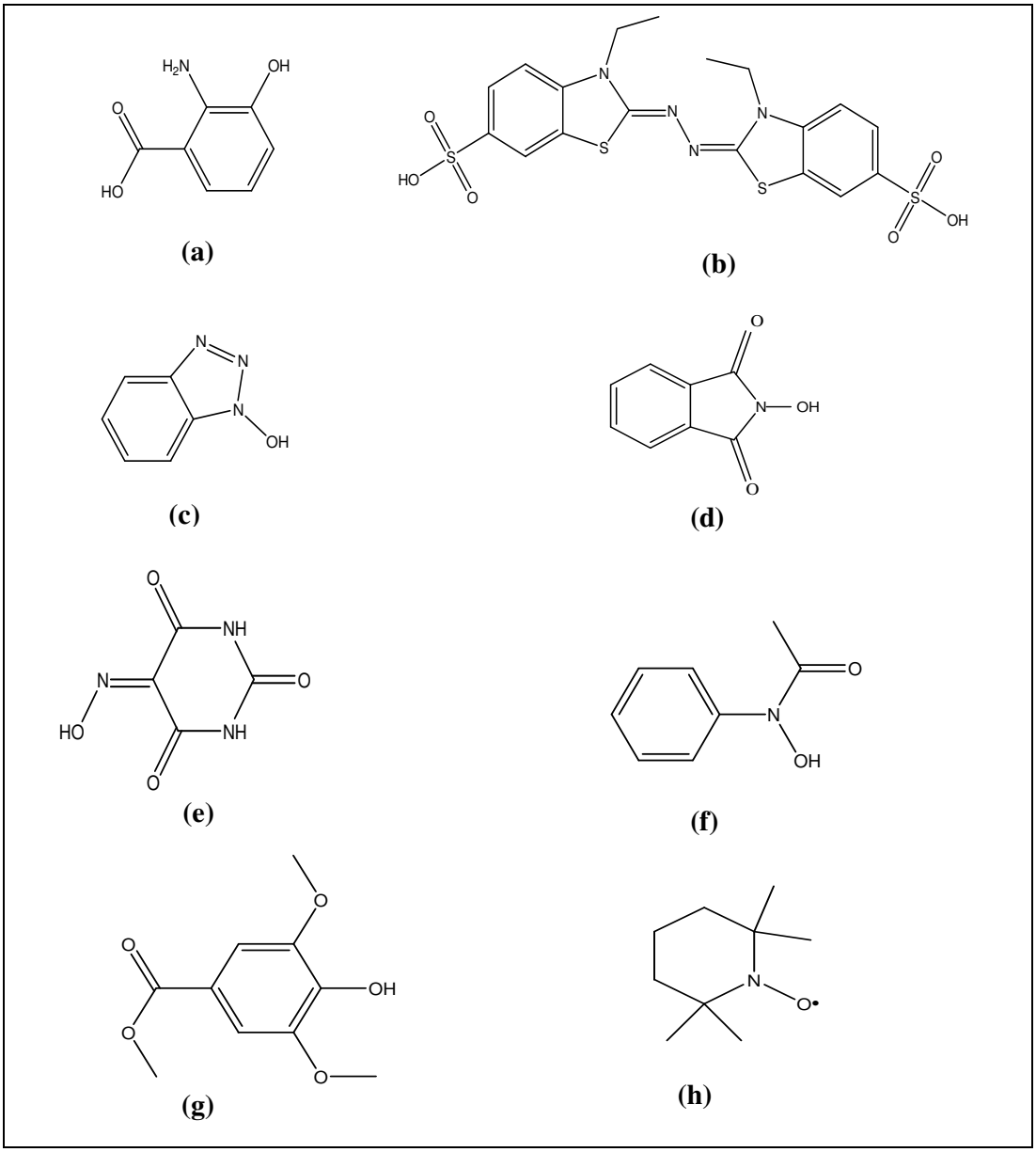

Fig. 3. Examples of laccases mediators. **(a)** 3-Hydroxyanthranilic acid (HAA); **(b)** 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS); **(c)** *N*-hydroxybenzotriazole (HBT); **(d)** *N*-hydroxyphtaimide (HPI); **(e)** violuric acid (VLA); **(f)** *N*-hydroxyacetanilide (NHA); **(g)** methyl ester of 4-hydroxy-3,5-dimethoxy-benzoic acid (syringic acid); **(h)** 2,2,6,6-tetramethylpiperidine-1-yloxy (TEMPO).

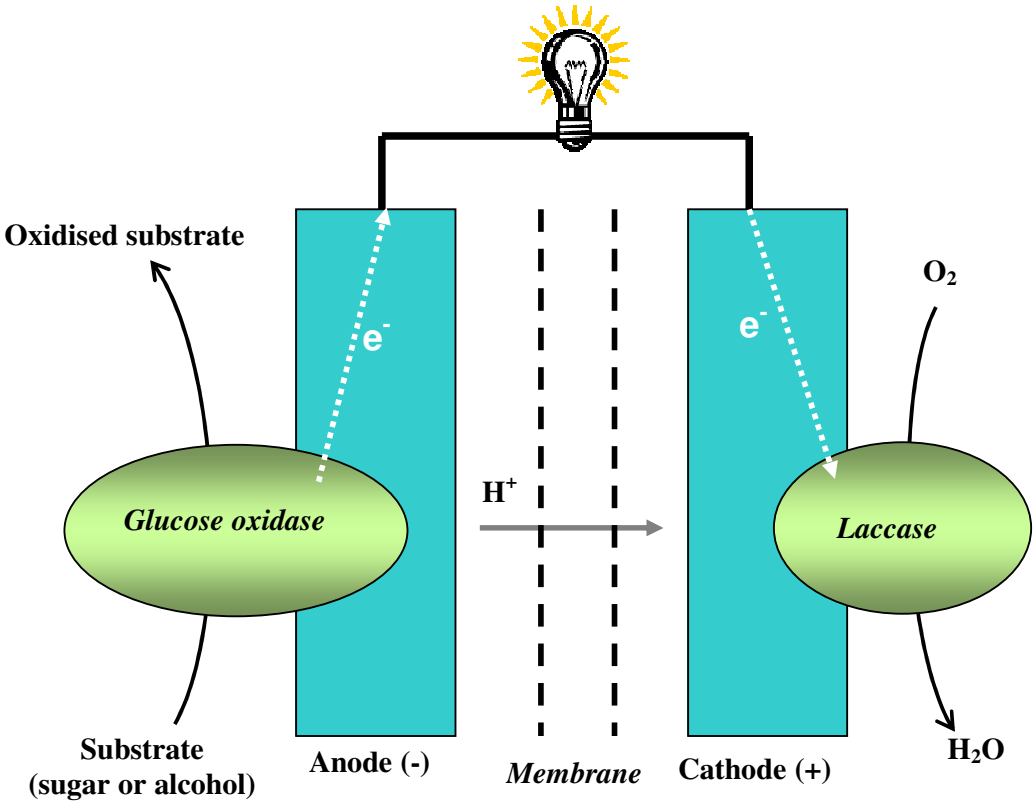

Fig. 4. Schematic representation of a biofuel cell involving glucose oxidase and laccase enzymes.