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A REVIEW ON THE POTENTIAL INDUSTRIAL APPLICATIONS OF MICROBIAL LACCASES

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ABSTRACT

Laccases are multicopper holding enzymes which can catalyze variety of reactions involving one electron oxidation. Laccases are listed ecofriendly because they involve molecular oxygen as final electron acceptor and thus produce water as by product. Laccases have capability to oxidize both phenolic and nonphenolic lignin related compounds as well as noxious environmental pollutants and thus derive interests and attraction from researchers to be used in industrial biotechnology. This enzyme is being utilized in a variety of industries from food processing to textile and paper industry. Ability of laccases to eliminate phenolic compounds is being utilized in ethanol production and genetically enhanced systems are being used for laccase production. Laccase also finds its application in nanobiotechnology and biomedicine. Their ability to transform xenobiotic compounds makes them efficient bioremediation agents. This review covers the scope and applications of laccases within different industrial fields.

KEYWORDS: Laccase, oxidoreductase, microbes, enzyme system, bioremediation.

INTRODUCTION

Laccases (EC 1.10.3.2, benzenediol: oxygen oxidoreductases) are multicopper oxidases and are part of the monomeric glycoprotein group.[1] Oxidation of amino-phenols, ortho and para di-phenols, polyamines, aryl diamines, lignins and poly-phenols as well as some inorganic ions along with the reduction of molecular dioxygen to water is catalyzed by this enzyme.[2] Laccases are produced by fungi (mostly white rot fungi)[3], higher plants[4] and bacteria.[5] Lichens[6] and sponges[7] have also been found to produce this enzyme. The biological function of laccases can be speculated by knowing their origin of production: laccases produced in fungi are engaged in stress defense, morphogenesis, degradation of lignin and resistance against fungal pathogen[8]; those found in bacteria are involved in pigmentation, morphogenesis, toxin oxidation and protection against oxidizing agent and UV light.[9]; laccases of plants participate in wound responses and lignin polymerization.

The 3D structure of laccases has been studied in many experiments and has been discussed in many reviews. The structure is held up by monomeric units consisting of three domains which are arranged in a sequence to form a barrel type structure. In recent studies, it has been reported that fungal laccases lack high order oligomeric assemblies making the crystal lattice and are found generally as monomers. As far as the laccase mechanism of action is concerned, there are two individual sites that bind the reducing substrate and O2 with four catalytic copper atoms: the paramagnetic type 1 copper (T1Cu) which imparts characteristic blue color of the protein in the reduced resting state, where the substrate oxidation takes place; the T2Cu and the two T3Cu that are clustered 12 Å away from the T1Cu as shown in the figure 1. This trinuclear cluster is where O2 is reduced to two molecules of water, receiving four consecutive electrons from four independent mono-oxidation reactions at the T1Cu site through a strictly conserved His-Cys-His electron transfer route.[10]

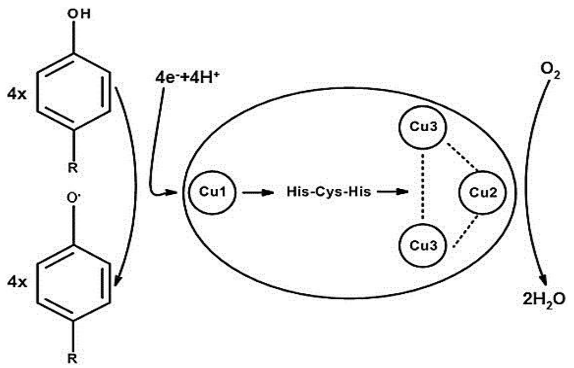

Figure 1: Schematic diagram of laccase catalytic cycle. O_2 is reduced to two water molecules in the trinuclear cluster of copper atoms.

Laccase cannot oxidize non-phenolic compounds due to presence of lower redox potential in this case. However, studies show non-phenolic structures can be oxidized by laccases in presence of electron transfer mediators.[11] Although industries find oxidation reactions very reliable, most of the conventional oxidation technologies have the following flaws: non-specific or unwanted side-reactions and involvement of environmentally toxic chemicals. This problem led to the search for new oxidation technologies based on biological systems such as enzymatic oxidation. Enzymatic application has following advantages over chemical oxidation: enzymes have specificity for a particular substrate to be biodegraded and enzyme reactions are carried out in mild conditions. The potential applications of laccases have been broadly reviewed in recent years. These reviews discuss the use of laccase mediated systems in food industry[12], in pulp and paper industry[13], in forest product industry[14], in grafting reactions[15], in bioremediation[16, 17] and in organic synthesis.[19,20,21] Also, there are many reviews in which the role of engineered laccases in biotechnology industry has been argued.[22]

ADVANCED ROLES OF LACCASES IN DIFFERENT FIELDS OF INDUSTRIAL BIOTECHNOLOGY

During the last decade, there has been a huge rise in the concern for using laccases as biocatalysts instead of traditional chemical catalysts in industries like textile, pulp and paper and pharmaceutical. Laccases also have applications in cosmetic, paint and furniture industries. Laccases have also been used in the production of bioethanol from lignocellulose materials. The biotechnological use of laccases nowadays is a booming research area as shown in Table 1.

1. Commercially available laccases include laccases expressed in *E. coli* in heterologous way, as well as laccases produced in lacquer tree *Rhus vernicifera*, in filamentous fungi (*Aspergillus spp*.) and from many bacidiomycete species including *Agaricus bisporus*, *Cerrena unicolor* and *Trametes versicolor*. The lucrative commercial products of laccases include color enhancement in tea, pulp bleaching and finishing and cork treatment.[23, 24]

Table 1: Potential Applications of Laccases

S.No.	Potential Application	Associated Reaction	References of Related Reviews
1.	Textile industry	Oxidative delignification (mainly using LMS) and oxidative degradation	[15, 23, 25]
2.	Paper and Pulp Industry	Oxidative delignification (mainly using LMS)	[23, 25, 26, 27]
3.	Wood Products	Coupling and grafting and co-polymerisation	[14, 15]
4.	Organic Synthesis	Oxidative oligomerisation	[19, 20, 21, 23, 28]
5.	Bioremediation	Oxidative degradation (usually in the presence of mediators); coupling coupled with Oxidative polymerisation, demethylation or dechlorination; and oxidative cleavage	[15, 23, 25, 27, 29, 30]
6.	Biosensors	Oxidation	[23, 25]
7.	Pharmaceutical Industry	Oxidative oligomerisation	[15, 20, 23, 25]
8.	Food Industry	Coupling/crosslinking reactions	[93, 12, 15, 23]

Food Industry

As mentioned earlier, laccases can catalyze homo and hetro-polymerization reactions. This property of laccases is utilized in food industry by applying them in wine and beer stabilization, fruit juice processing and sugar beet pectin gelation. The organoleptic properties of wine can be maintained by removing the unwanted phenolic compounds using laccases.[12] These phenolic compounds are present in high concentration and affect taste, color and gustative sensations of wine. Furthermore, treatment of cork stoppers for wine bottles is being done by commertially produced *M. thermophila* laccase. This process involves the oxidation of phenols and non-enzymatic homo-polymerization of resulting phenoxyl radicals averting the formation of 2,4,6 trichloroanisole which is responsible for cork taste. The cork's surface is also modified by this process which increases its hydrophobicity and diminishes the extraction of substances in wine.[31] The ability of laccases to crosslink biopolymers is currently being utilized in baking. It has been shown that laccase from white rot fungi *Trametes hirsute* can decrease the extensibility of both flour and gluten dough and can increase the maximum resistance of dough. The color and flavor of teas and oil containing products have also been shown to be enhanced by laccases.[32]

Oxidation of phenolic compounds in fruit juices has adverse effects on the organoleptic properties of juices.[12] To preserve and to keep the juices in stable form, the formation of polyphenol-proteins is delayed and there are many studies which show that this stabilization can be carried out by laccases. In some cases, the removal of high concentrations of polyphenols in fruit juices was carried out by laccases which was much efficient process than using traditional stabilizers.[33] Many studies show that the treatment of fruit juices with laccase in conjunction with a filtration process can improve colour and flavour stability.[34, 35, 36, 37]

In recent studies, low-cost carriers for immobilization of laccase have been applied in the clarification of fruit juice.[38] Specifically, laccase from *T. versicolor* was immobilized in coconut fibres (CF) activated with glutaraldehyde. The laccase-glutaraldehyde-CF matrix was utilized to clarify apple juice, lightening the original juice colour by 61% and removing 29% of its turbidity. In a recent project, a recombinant POXA1b laccase from *Pleurotus ostreatus* was immobilized on epoxy activated poly(methacrylate) beads and tested in the clarification of several fruit juices, producing a reduction in phenol of up to 45%.[39] Moreover, the laccase-treated juice had comparable flavanone content to the non-treated juice but a dramatic reduction in vinyl guaiacol (an off-flavour with a pepper-like aroma).

Many reviews discuss the potential applications of laccase in different forms of the food industry such as bioremediation, beverage processing, ascorbic acid determination, sugar beet pectin gelation, baking and as a biosensor. Nonetheless, low cost production and extraction systems of laccases are much needed.

Paper and pulp industry

In paper industry, lignin is extracted from wood pulp and then degraded for further processing. Environmental protection protocols demand to replace conventional and polluting chlorine-based delignification/bleaching procedures. Thus, for decades, there have been attempts to replace these conventional chlorine-based delignification processes with cleaner and milder strategies, paying special attention to the pre-treatment of wood pulp with ligninolytic oxidoreductases. The benefit of using laccases instead of peroxidases is that laccases require O_2 rather than H_2O_2 and also their ability don't gets affected by co-substrate unlike peroxidases. It is interesting to know that a LMS is currently marketed to increase throughput in mechanical pulping, to enhance paper durability and to reduce pitch problems.

In recycling paper, the process of removing flexographic inks is very important. To provide alternative and bio-based deinking methods, laccases from the ascomycete *M. thermophila* and from the basidiomycetes *Trametes villosa*, *Coriolopsis rigida* and *Pycnoporus coccineus* were assessed for effect of synthetic and natural mediators on discoloration of flexographic inks.[40] The three basidiomycete laccases had better decolourization capacities than the laccase from *M. thermophila*, enhancing decolourization by applying natural and synthetic mediators (especially HBT). Certainly, only the lignin-derived mediators acetosyringone and methyl syringate were able to decolourize all the inks assayed with *M. thermophila* laccase, although all activity was lost after four hours.

Recently, use of laccase for grafting of phenols to flax fibers for the production of paper has been studied.[40, 41] However, the processing of flax and sisal pulps with laccases from *P. cinnabarinus* and *T. villosa* was studied with involvement of different phenolic compounds.[41] In many studies, laccase usage led to the covalent incorporation of the phenols into the fibres, with the highest rate of phenol grafting recorded when p-hydroxycinnamic acids, p-coumaric and ferulic acids were applied. In other studies, the treatment of unbleached flax fibres with laccase from *P. cinnabarinus* and low-molecular weight phenols was checked.[40] Paper handsheets from pulps processed with laccase and phenol were assessed for their antimicrobial and optical properties, showing antimicrobial activity against three bacterial species evaluated (*Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*), as well as a decrease in brightness and an increase in coloration.

Textile industry

Major applications of dyes are in textile sector and textiles demand usage of large amount of water and chemicals for processing. There is a variety of chemicals, which are used in textile processing, including inorganic chemicals and organic compounds. Laccases have gained importance to be used in textile industry for bleaching processes of textiles[42, 43] and they have now replaced peroxide bleaching for enhancing the whiteness of cotton.[44] Laccase products are now being commercialized all over the world and being applied for denim bleaching.[45] Aromatic compounds can be oxidized by laccases to form colored products. Using this characteristic of laccases, they are being used to produce different textile dyes like phenoxazine and azo dyes.[46] In recent studies, laccases have been utilized to dye cotton and wool fabrics with heteropolymeric dyes which are produced when precursors generated by laccases undergo oxidative hetero-coupling.[47] In an impressive study, substituted phenoxazinones and phenazines dyes were synthesized from o-phenylenediamines, substituted p-phenylenediamines and o-aminophenols using CotA laccase from *Bacillus subtilis* and the laccase from *T. versicolor*.[48] The initial aromatic amines were first assessed electrochemically and oxidized by laccase with unusual yeilds when observed on a calliberated scale, giving rise to novel phenazine and phenoxazinone dyes.

Laccases are also used in cloth washing detergents as they are effective against odour in fabrics.[23] In a study, shrinkage of wool was found to be reduced when laccase mediated system was applied.[49] Laccases have also been applied as a graphting bio-catalyst in processing of wool fabrics. This study opens up new possibilities for the development of multifunctional textile materials with antibacterial, antioxidant and water repellent properties.

Biofuels

One of the most reliable feedstocks for the production of bioethanol are lignocellulosic materials. However, their usage relies on the efficient hydrolysis of polysaccharides, which requires a low-cost pre-treatment of biomass to eliminate lignin and expose sugars to hydrolytic enzymes. Laccases have a major role in lignin biodegradation, and thus, their potential utilization as catalysts in production of biofuel is under research, and as agent to remove yeast growth inhibitors (mainly phenolics) for the subsequent enzymatic processes. A laccase from *T. versicolor* heterologously expressed in *Saccharomyces cerevisiae* enhanced the production of bioethanol through eliminating phenolic compounds.[50] In a recent study, a newly identified laccase from the white-rot fungus *Ganoderma lucidum* was tested to produce bioethanol and to remove toxins in lignocellulosic hydrolysates. This laccase removed 84% of the phenolic content in corn stover hydrolysate, and when applied prior to cellulose hydrolysis, it improved ethanol yield by 10%.[51] Figure 2 summarizes the potential biorefinery applications of laccase mediated systems.

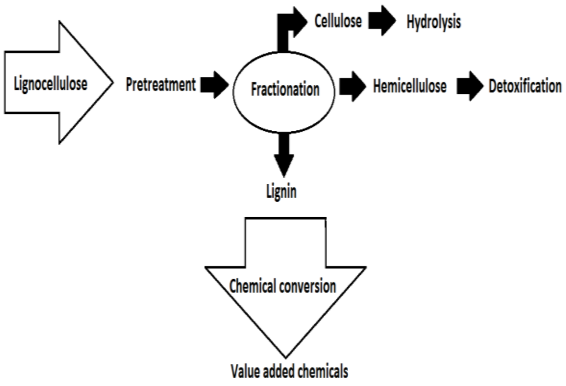

Figure 2: Possible areas of application for laccases in biorefinery processes. The three potential biorefinery applications of laccase mediator systems are (1) biomass pretreatment, (2) lignin conversion and (3) hydrolysate detoxification. After pretreatment, the carbohydrate fractions can contain lignin-derived phenols potentially inhibiting cellulose hydrolysis as well as sugar fermentation which have to be removed by a detoxification step.

A new interest in this developing field is the engineering of a full consolidated bioprocessing microbe (by engineering an artificial secretome in yeast) that possess the key enzymes of the ligninolytic consortium.[52, 53] Table 2 shows different modes of action and microbial sources of laccases which are applied in biogas and bioethanol production.

Table 2: Applications of laccases in the production of bioethanol and biogas

S.No.	Substrate	Source of Laccase	Mode of Action	Reference
1.	Eucalyptus	*Myceliophthora thermophila*	Delignification	[54]
2.	Wheat straw slurries	*P. cinnabarinus*	Polymerization and reduction of phenolic content	[55]
3.	Wheat straw	*P. cinnabarinus*	Polymerisation and removal of phenolics	[56]
4.	Wheat straw	*P. cinnabarinus*	Removal of phenolic compounds	[57]
5.	Cotton jin trash	Modified strains of *Cerrena unicolor*	Delignification and modification of cellulose structure	[58]
6.	Paddy straw	*Streptomyces griseorubens ssr38*	Delignification	[59]
7.	Newspaper	*Trametes sp.*	Delignification/lignin modification; de-inking	[60]
8.	Corn stover	*T. versicolor*	Delignification	[61]
9.	Comcob residue	*Trametes sp.* AH28-2 heterologously expressed in *Trichoderma reesei*	Delignification (probable; not investigated)	[61]
10.	Rice hull	*Pleurotus ostreatus*	Delignification	[62]
11.	Japanese cedar wood	*Ceriporiopsis subvermispora* ATCC 90467, CZ-3 and CBS 347.63	Delignification	[63]
12.	Wheat straw	*Coriolopsis rigida* and *T. villosa*	Polymerisation of phenolic compounds	[64]
13.	Steam-pretreated softwood	*Trametes hirsuta*	Delignification (partial)	[65]

Nanobiotechnology

Laccases have the ability to catalyze reactions by direct electron transfer and therefor scientists are taking interest in developing biofuel cells and biosensors based on laccase mediated systems.[66] In case of biosensors, the oxygen usage during analyte oxidation is assessed by biosensor when O_2 is reduced to H_2O by laccases. Biosensors based on laccases are broadly used in food industry to keep check of polyphenols in fruit juices, wines and teas and to estimate contamination of fungus in grape musts.[67] Biomedicine industry also utilize laccase based biosensors for analysis of insulin, morphine and codein.[68]

One very impressive utilization of HRPLs is their use as cathode enzymes in biofuel cells for biomedical purposes[69] (Fig. 2). A research group was recently successful in processing out the laccase from the basidiomycete PM1 (E00 T1 = +759 mV versus NHE) to produce a laccase active in human blood.[70] This blood tolerant laccase was then adjusted into a self-powered and wireless device, opening new avenues for applications not only in medical lab technologies but also in analysis of environmental toxins, micro-process technologies and bio-computing.[71] On the other hand, this laccase modified strain was also attached to a low-density graphite electrode for the oxidation of water at pH > 7, regressing the natural activity of the laccase and opening an exciting area of research into water splitting.[72]

A great interest in nanobiotechnology nowadays is the utilization of implantable biofuel cells which can obtain power from natural sources. In recent years, preliminary results were published of an enzyme biofuel cell working in an orange in vivo.[73] Catagorically, the biofuel cell involved catalytic electrodes with glucose dehydrogenase and fructose dehydrogenase immobilized on the anode and with laccase from *T. versicolor* on the cathode. The cathode/anode pair was incorporated in orange pulp, deriving power from its content (juice contains glucose and fructose). Then this power generated from the orange was used to supply a wireless electronic system. Biomedical and biomaterial research nowadays has its direction in the development of polymers with bioresponsive properties to detect potentially pathogenic microorganisms. Bioresponsive hydrogels based on carboxymethyl-cellulose and peptidoglycan were developed to mark lysozyme in infected wound fluids and cellulases secreted by potentially pathogenic microorganisms, respectively.[74] A laccase from *Trametes hirsuta* was chemically manipulated with polyethyleneglycol or methacrylic groups, and it was incorporated into the hydrogels to carry on the signal and the stability relative to simple dye release-based systems.

Organic synthesis

In pharmaceutical industry, laccases play an important role as it has the ability to catalyze a broad range of synthetic reactions ranging from the transformation of antibiotics to the derivatization of amino acids for the synthesis of metabolically stable amino acid analogues.[24] Indeed, laccases have been utilized to synthesize complex medical products, like anti-cancer drugs (e.g. vinblastine, mitomycin and actinomycin), immunosuppressors (e.g. cyclosporin A) and antibiotics (e.g. penicillin X dimer and cephalosporins).[23] Moreover, laccases have been applied to oxidize the steroid hormone 17b-estradiol and stilbenic phytoalexin trans-resveratrol, producing dimers or oligomers after coupling of the radical intermediates.[75, 76] In addition, they have also been used for deriving amino acids such as L-tryptophane, L-phenylalanine or L-lysine through enzymes.[77]

Catechin polymers can be utilized to weaken postprandial hypercholesterolaemia and hyperlipidaemia, and their synthesis can be catalysed by *M. thermophila* laccase, affecting lipid and cholesterol absorption.[78] Laccase-catalyzed catechin polymers have more enhanced inhibitory activity against pancreatic lipase and cholesterol esterase than the catechin monomer. Another potential utilization of laccases is in the oxidation of iodide to generate iodine (I_2), a low-cost and efficient antimicrobial compound.[79] During a recent project, the laccase-assisted synthesis of I_2 based on an artificial neural network was studied with a genetic algorithm.[80]

Dimeric phenols enriched with enantiomers are now being developed by using laccases and lipases and these phenols resemble in structure with b-5 dimers of lignin.[81] Different studies show that laccases can be very effective for alcohol oxidation when used with palladium catalysts.[82] Categorically, the LAC3 laccase from *Trametes* sp. C30 was applied with four different water soluble palladium complexes which possess capability to oxidize primary and secondary alcohols under harsh conditions (high temperature and pressure). The laccase-palladium complexes were then assessed for the aerobic oxidation of veratryl alcohol into veratryl aldehyde at room temperature and atmospheric pressure. Eventually, the efficiency of the complex (laccase and palladium-II) to catalyze the reaction improved up-to many folds.

Cosmetics

Many personal care products have been developed by cosmetic industry through exploiting the oxidative potential of laccases. While cosmetic and dermatological formulations containing laccases were patented for skin lightening.[83], also in the field of hair bleaching and dying, laccases have wide applications. The bleaching and dying of hair usually require the use of harsh chemicals like H_2O_2 that can cause harm to hair and damage the scalp.[84] Laccases can be applied to replace H_2O_2 as oxidizing agent in the preparations of hair dyes. Recently, novel laccases from the actinomycete *Thermobifida fusca* and from the basidiomycete *Flammulina velutipes* have been assessed in the oxidation of dye intermediates broadly used in hair-coloring.[85] In addition, a hair color was recently developed composed of butein and either a combination of a peroxidase with either H_2O_2 or a H_2O_2 generator or a laccase.[86]

Paints

The polymerization of poly alcohols, dicarboxylic acids or anhydrides and unsaturated fatty acids produce Alkyd resins.[87] These resins are chiefly utilized as binding agents in coatings, although they are also used as road materials, house and decorative paints. Cross-linking of the unsaturated fatty acids aided by heavy metals is involved in chemical drying of these resins. Nowadays, techniques to replace heavy-metal-based catalysts with less toxic and environmentally friendlier alternatives are under consideration. A recent study showed that that LMS can effectively replace heavy metal catalysts and cross-link the alkyd resins.[88] It raised the curiosity of researchers as the biocatalytic reaction worked both in aqueous media and in a solid film.

Furniture

Cross-linking of lignocellulosic materials with a synthetic resin in heat pressure under moisture produces dry formed panel products called Medium-density fiber boards (MDF).[89] They are used to construct a broad variety of furniture like wardrobes, cupboards, tables, desk tops, TV tables, beds and sofas. Traditional MDF manufacturing involved non-enzymatic cross-linking by applying toxic compounds like formaldehyde. Nowadays, considerations about formaldehyde emission toxicity and the high cost of petrochemical resins have created an awareness of using enzymatic binder systems as eco-friendly alternatives to glue lignin-based materials. LMS has been used to activate lignin on wood fiber surfaces in the pilot-scale production of MDF.[90] Moreover, a hot-air technique for the production of wood fibre insulation boards was reported that utilizes an LMS as a naturally based bonding system.[91] Laccases in combination with some glue have also been found to be effective in making wooden boards stronger and it enhanced its quality.

Bioremediation through Laccases

Xenobiotic compounds like polycyclic aromatic hydrocarbons (PAHs), phenols and organophosphorus insecticides are environmentally unsafe and are considered to have teratogenic and carcinogenic effects. These resisting chemicals make one of the major contaminants of soils and waters, and accordingly, their removal is a preferred task for most environmental agencies.[16] Apart from microbial bioremediation, the use of laccases in enzyme bioremediation either used with redox mediator factors or not has gained much importance. Generally, laccase or LMS can oxidize the xenobiotic to release a less toxic product with more bioavailability, which can be more efficiently vanished by physical and mechanical procedures. For example, the removal of PAHs like anthracene or benzopyrene[92], recalcitrant dyes like Reactive Black 5 or crystal violet[93, 94] and organo-phosphorous compounds, like the nerve agents VX or Russian VX.[95] Moreover, oestrogenic hormones present in effluents from sewage treatment can be oxidized by laccases. As a fact, the oxidation of the oestrogens estrone, 17b-estradiol and 17a-ethynylestradiol by a fungal laccase from *Trametes* sp. Ha1 has been reported.[96] Moreover, a treatment system was developed that comprised the laccase and a b-D-glucuronidase to degrade the 17b-estradiol 3-(b-Dglucuronide), efficiently removing this compound and its intermediate 17b-estradiol.

CONCLUSION AND FUTURE PROSPECTS

Laccases are being studied since 19th century and one might think it as old fashioned enzyme but currently the status of laccases are different. It is considered as the most effective green catalyst of present time by many researchers. Many types of industries are utilizing laccases for modification and processing of their product like paper and pulp, textile, cosmetic, furniture, biomedicine and food industry but still there are areas in laccase biotechnology which are still to be explored. Different companies are producing and utilizing laccase while applying conditions like choice of organism which promote maximum output. Many protein engineering techniques have been applied to enhance output but still there is not a trend of using laccases at large industrial scale. One of the biggest hurdle in commercialization of laccases is lack of sufficient enzyme stocks. Another hurdle is the high costs of mediators. This problem can be encountered by using naturally derived compounds as mediators. Laccase encapsulation with polyelectrolytes will be used as a microreactor for catalytic reactions by changing the permeability properties of the capsule wall. Different immobilization modifications are also under study. Also, strategies like protein engineering will expand the variety of laccases and open up new avenues in biotechnology.

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NIL.

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