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# Determination of phenolic compounds with recent development in Laccase Biosensor

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**ABSTRACT:** Laccase belongs to the multi copper oxidase family that catalysis the one electron oxidation of phenolic compounds. For catalysis of phenolic compounds it requires oxygen as co-substrate and it yields water as by-product. Laccase is distributed in fungi, bacteria and higher plants and also reported in lichens and sponges. Laccase was first discovered in Japanese lacquer toxicodendron vernicifera (Rhus vernicifera) in 1883. Laacase contains three type of copper atoms in which $T_1$and $T_2$ is paramagnetic and $T_3$ is diamagnetic. $T_1$is the first active site for catalysis (substrate oxidation site) and $T_2$and $T_3$ forms a trinuclear cluster where molecular oxygen is reduced to water. Many methods are used for immobilization of laccase such as direct adsorption, physical entrapment, conjugation and covalent attachment. Laccase enzyme is used for construction of biosensors for determination of various compounds such as tartrazine, phenols in wastewater, catechol, detection of Bisphenol A, polyphenol index in wine, xenobiotics.

## 1. INTRODUCTION

Laccase is one of the oldest enzymes. In 1883, Yoshida described their occurrence in the Japanese lacquer tree toxicodendron vernicifera. Laccase are typically extracellular monomeric glycoprotein belongs to the multicopper oxidase family. Laccase (EC 1. 10, 3. 2 benzendoil; oxygen oxidoreductase) catalyse the oxidation of oxygen to water (Torrecilla et al., 2007). Laccase are mullticopper containing enzyme which reduce molecular oxygen to water and simultaneously perform one electron oxidation of various substrates such as diphenols, methoxy-subsituted monophenols and aliphatic amines application(Kudanga et

al., 2011). Three types of copper atoms are present are in which $T_1$ and $T_2$ are paramagnetic and $T_3$ are diamagnetic. Laccase is used in various processes including bioremediation chemical synthesis, bio bleaching of paper pulp, bio sensing, textile finishing, wine stabilization. Laccase is determined by their origin and the stage of life of the organism producing them. In the stress defense, morphogenesis, fungal plant pathogen/host interaction and lignin degradation, fungal laccase is used. In pigmentation, morphogenesis, toxin oxidation and protection against oxidizing agents and ultraviolet light, bacterial laccase is involved. In wound responses and lignin polymerization, plant laccase are involved. Area of laccase is increasing because they have a wide substrate range. The catalytic properties of laccase had a great impact on the development of biosensor (Palanisamy et al., 2017). Laccase are encoded by multigame families in all higher plants. In poplar plant, we have characterized 5 distinct laccase.

Many laccase are used to detect phenolic compounds. Polyphenol Oxidase(PPO) is used as a biosensor, it shows great advantage include ability of polyphenol oxidase to catalyze electron transfer reaction without need of additional cofactors, oxidation of phenolic compounds in the presence of oxygen(Gul et al., 2017) Polyphenol oxidase catalyzes the oxidation of phenols to o-quinones, which are highly reactive. For detection of phenolic compounds, a number of biosensor have been reported which is based on immobilization of laccase onto various supports such as sol gel film, chitosan film, graphiteelectrode, polyethrtsulfone membrane, nafion sol gel silicate, cellulose-based granocel and woven nylon supports through adsorption. An enzyme is commonly required to be immobilized on or in close proximity to the surface of transducer, for reusable enzyme-based biosensor. The Immobilization of an enzyme is the attachment of the enzyme to an insoluble support. Immobilization of laccase leads to increase in the thermo stability of the enzyme. Physical or chemical interactions are the two basic methods for enzyme immobilization. Physical method involves the entrapment (such as polyacrylamide, collagen, alginate or gelatin is used) or encapsulation in an organic or inorganic polymer, whereas chemical method involves adsorption and covalent binding or self-immobilization. Adsorption is based on

ionic and other forces of attraction. The ionic strength of the medium and the pH and the hydrophobicity of the support surface must be noticed during immobilization of enzymes. Covalent binding is the activation of the chemical groups on the support surface and react with nucleophile groups on the protein. Many types of support system are used for activation of chemical groups including silica-based supports such as kaolinite or mesoporous silica nanoparticles. Many types of electrodeshave been designed to act as suitable support for laccase immobilization.

## 2. BIOSENSOR

A biosensor, according to the international union of Pure and Applied Chemistry, is a self-contained integrated receptor-transducer device that uses a biological recognitionelement (biochemical receptor) that is kept in direct spatial contact with an electrochemical transduction element to provide specific quantative or semi-quantative analytical information. A biomolecule is a device that detects biological molecules.(Gul et al., 2017)

According to the mode of physiochemical transduction or the type of bio recognition, biosensor can be classified into many types:Electrochemical biosensor, optical biosensor, piezoelectric biosensor**.** The detection of light absorbed or released as a result of a biological reaction is used in optical biosensors. These biosensors use a variety of optical techniques, such as adsorption, fluorescence, luminescence, surface Plasmon resonance (SPR) and so on.

The mass change that occurs as a result of bio molecular contact can be measured usig a piezoelectric biosensor. The mass cjange is measured using piezoelectric crystals by comparing the change in oscillation frequency of the piezo crystals.

Biosensors are categorized as enzymatic biosensors, nucleic acid biosensors, and whole cell based biosesnors based onn bio recognition. Compounds 2-4 D, phenol, catechol, polygala, 2-chlorophenol and4-nitrophenol were used to test biosensor activity.

## LACCASE ENZYME

Laccase is a multifunctional enzyme that catalyzes an oxidation process followed by a four electron reduction of molecular oxygen to water. It represents the largest group of multicopper oxidase(Fernández-Fernández et al., 2012)Their ability to oxidize phenolic compounds and their applications in theseveral industrial sectors have been studied in nineteenth century as of late ( (Favero et al., 2015; Sezgintrk et al., 2010).

Laccase based voltametric/aerometric biosensors have been developed for food and beverage analysis(Mate & Alcalde, 2017). These biosensors have foundwide application in the analysis of phenol, poly-phenol, Gallic acid, caffeic acid, catechin, catechol, hydroquinone and resorcinol to name a few in various food and beverage product(Nazari et al., 2015). Laccase can catalyse the oxidation of ortho, Meta, para-quinones or radical species. In these reactions, the oxygen is reduced directly to water without intermediate formation of hydrogen peroxide (Mazlan et al., 2017).

## OCCURRENCE OF LACCASE ENZYME

Laccase was first discovered in the Japanese lacquer treeRhus vernicifera(Giardiana et al., 2010; Morozova et al., 2007b). Fungal laccase are multicopper oxidases with high catalytic versatility and low requirement (only $Co_2$ of the air is required for activation). In addition to fungal laccases, bacterial laccases from Pseudomonas putida F6, Pseudomonas speciess LBC1,, and Escherichia coli have been isolated and described(Swetha m et al., 2011). Fungal laccases are typically extracellular proteins with varying degrees of glycolysation, whereas bacterial laccases are intracellular or periplasmic enzymes (Diana M. Late et al., 2014). Laccase play diverse biological diole that are determined by their origin and the life stage of the organism that produce them (Miguel Alcade., 2014).

## 3. CATALYSIS OF LACCASE ENZYME

Laccase catalysis the oxidation of a wide range of substrates including mono-, di-, and polyphenols, aminophenol's, methoxyphenols, aromatic amines and acerbate, while also reducing oxygen to water by four electrons (Madhavi and lele, 2009). These enzymes

couples the four single electron oxidation of the reductive substrate to the four electron reductive cleavage of the dioxygen bond with four electron copper atoms(Fernández-Fernández et al., 2013). The copper atoms are classified into three groups, depending on the characteristics obtained by ultraviolet/visible and electron paramagnetic resonance (EPR) spectroscopy. The type 1 coppe($T_1$) isparamagnetic and is strongly absorb at 600nm and is electron paramagnetic resonance detectable because of the charge transfer from the neighboringsulfur from acysteine and this gives the whole enzyme a blue color. The type 2 copper ($T_2$) is colourless but its paramagnetic but EPR (electron paramagnetic resonance detectable.The type 3 copper ($T_3$) is made up of two copper atoms that have a mild adsorption near the UV spectrum and no EPR signal is detectable.$T_1$ has highestredox potential andit rangesfrom 0. 5 to 0. 8 volt depending on the enzyme and this is our substrate oxidation site.Itis coordinated by two conserved histamine and one cysteine, the fourth ligand is variable and this can be either methionine, leucine, or phenylalanine and the fourth residue actually determine the specific redox potential of an enzyme. The $T_2$ and $T_3$ from a three nuclear cluster coordinated by several histidine and this is site where molecular oxygen is reduced to water.The architecture of the site is conserved and the distance between $T_1$ copper atom and the pronuclear cluster is always around 12 Angstroms. Ina catalytic cycle the substrate will bind to the pocket next to the type 1 copper which then extracts one electron from the substrate and this electron is relayed through protein functional groups to the pronuclear cluster namely through the tile of the cysteine and then carbonyl and imidazole of the histamine. ((Fernández-Fernández et al., 2013)

## 4. ROLEOFLACCASEBIOSENSOR

### Forquantification of phenol in wastewater

Many phenoliccompounds have listed as prioritized hazardous pollutant by European Commission (EC) and United States Environmental Protection Agency (US-EPA) for their

presence in drinking water because of their toxicity and hazardous nature to human and animals.

According to EC, 0. 001 mg/$l^2$concentration limit is permissible. Laccase based biosensor are very useful in the food industry as well as in biomedical application for the detection of phenolic compounds. (Yashas S. et al., 2018). (Cabaj et al., 2011)developed a hybrid phenol biosensor of laccase by electrostatic deposition from cerrena unicolor on polythiophene films for the determination ofconcentration of phenol, o-aminophenol and catechol. (Sarika et al., 2014)designed laccase aerometric biosensor for industrial wastewater.

**Forthe determination of rutin**

Ionic iquids are the combination of organic cations and a variety of anions. Theconsiderable difference in the size of a cation and a small anion affects the packing of the lattice. Ionic iquids are the ions that melt at temperature below 100 C.

In the construction of carbon paste electrode, ionic liquids have been used as binder. For the determination of various substances, several modified CPE have been successfully developed. A CPE consist of non-conducting organic liquid, Nujol and paraffin and electrically conducting graphite powder is used. The modified carbon paste electrode have many advantages such as versatility, renewability, ease of construction, controlled bulky modifications and low background current. Polyphenol compound flavonoids rutin has anti-allergic, anti-inflammatory, anti-tumor and anti-bacterial effects. Several methods have been used for the development of sensitive, selective, simple and reliable method for determination of compounds including high performance chromatography, chemiluminiscense, spectrophotometry, capillary electrophoresis. For the determination of ruin in pharmaceutical formulations, a poly (vinylpyrrolisone)(PVP)-modified carbon paste electrode is developed. Rutin is absorbed on the surface on a modified electrode. A biomimetic sensor based on a dinuclear $Mn^{III}Mn^{II}$ complex has been reported for the determination of rutin. Polyphenol oxidase (PPO) is used as a bioelement in the development of biosensor for the amperometric detection of rutin. On an electrode surface,

PPO is entrapped within a laponite clay film. Addition of different amount of rutin (29. 1, 39. 9 and 45. 2 mg l¯) to 3 pharmaceutical samples was studied. By comparing the concentration obtained for samples with or without addition of known concentration of rutin standard solution. The percentage recoveries are shown in table. Due to low cost simplicity and fast construction of the biosensor, it is superior to others for determination of rutin.

**For Tartrazine**

Laccase is used to determinethe azo-dye tartrazine concentration. Tartrazine is an organic synthetic food dye. Tartrazine is found in beverages, candies, dairy products and bakery products. (Wang & Zhao, 2015). Due to azo groups (N=N) and aromatic ring structure contribution. (Mao et al., 2014) It is harmful for human being, so the content of tartrazine must be controlled. In china, only 0. 1g/kg (individual or in combination) tartrazine is permitted. (Wang & Zhao, 2015)More consumption of tartrazine leads to many health effects such as anxiety, migraine, allergies, diarrhoea and childhood hyperactivity(Gan et al., 2012)

(Mazlan & Hanifah, 2014)have reported the immobalization of laccase enzyme on poly (glycidyl methacrylate-co-n-butyl acrylate)(poly(GMA-Co-Nba)) microsphere and and a carbon-paste-screen-printed electrode is coated with a laccase conjugated microspheres and gold(Au) nanoparicles on the biosensor membrane. It has a hydrophobic property. Due to hydrophobic nature, the immobalization of laccase is confined to the surface, which allows the occurrence of the surface of the enzymatic reaction at the surface and elimination of diffusion limitation within the polymer matrix is done.

**Fordetection of xenobiotics**

Laccase are multicopper enzyme belonging to the group(More et al., 2011) of blueoxidase that catalyze the oxidation of a variety of aromatic campound. They acts as redox mediators and oxidize other compounds. A number of synthetic organic and inorganic mediators have been found (Shleev et al., 2006). In agriculture, chlorinated phenoxyacids are widely used

compound. For the determination of exogen xenobiotics 2, 4-D as well as some phenolic compounds, Trametes versicolor laccase was used to develop biosensor.

**For determination of polyphenol index in wine**

By using a flow system at a fixed potential of -100mV vs silver/silver chloride electrode in Britton-Robinson buffer 0. 2 mol $l^{-1}$ and pH 5, the amperometric measurements were carried out.Then, the results were compared with those obtained with Folin-Coicalteau reference method. For gallic acid, the biosensors show fast and reliable amperometric response with a linear range 0. 1-18. 0 mg $l^{-1}$for this detection, two different types of laccase Trametes versicolor (TvL)and Trametes hirsuta (ThL) was used.

**For Bisphenol A detection**

Electrochemical biosensor provides rapid and on-site BPA detection. For this, tyrosinase is used as a biological element for development of electrochemical biosensor and different immobilization procedures (Favero et al., 2015). In this paper, biosensor for BPA detection based on laccase coupled with disposable sensor. The effect ofthionineas a mediator hs been investigated when the immobilized laccase interact with BPA, by using cyclic voltammetry (CV). For increasing the performance, a screen printed electrode (SPE) modified by usig a nanocomposite formed by carbon black and thionine has been used. In the term of immobililzed enzyme units, applied potential and pH, the biosensor response was examine. CV measurement using thiamine in solution. In order to study the electrochemical properties of thionine towards BPA/laccase reaction products, preliminary experiments were performed. Thionine is an artificial organic dye which is derivate of phenothiazine. It is use as a mediator. Its formal potential falls between 0. 08 and -0. 25 V. After pouring thionine on SPE, the typical thionine voltammorgram obtained. After addition, of BPA/laccase reaction product, changes in voltammgram is easily noticeable. Due to thionine reduction process the curve signal increases in respect to the one in the absence of BPA/laccase reaction product. Conversly, due to thionine oxidation process, the anodic peak of current signal decreases, as expected for mediator reaction. The anodic peak is proportional to the

amount of thionine$_{red}$ and after the enzyme/BPA reaction. This quantity decreases.This demonstrates that thionine has an electrocatalytic action in reducing BPA/laccase oxidation products.

**BPA concentration determination**

In term of enzyme loading (1. 19U), pH (0. 05M citate buffer at pH4. 5) and applied potential (-200 mV), the biosensor response is optimized, then the signal current was studied as a function of BPA concentration. In terms of enzyme loading, pH and applied potential, the response of the biosensor was optimized, reaching a detection limit of micromolars level. When compared with laccase carbon paste biosensor(Matsumura et al., 2015) and tyrosinase-thionine glassy carbon electrode (Dempsey et al., 2004), the biosensor shows better results. The coupling of thionine with nanostructured materials (such as carbon black), leads to the development of a stable, miniaturized and low cost device for BPA detection.

**For detection of Catechol**

Catecholis a benzenediol with a benzene core and two orthogonal hydroxy substituents, has been categorized as a periodic hazardous pollutants by the US Environmental Protection Agency and the European Union due to its low biodegradability and severe toxicity on human health and ecosystem.The chromatographic methods are highly sensitive towards CC, but when compared with electrochemical methods, they are required sample pretreatment, expensive, not portable and often time consuming. As compared to non-enzymatic, CC sensors (Karim, F. & Fakhruddin, A. N. M., 2012), enzyme based biosensors are highly sensitive towards CC. As a result,the enzyme based biosensor has gotten a lot of attention for detecing CC in a selective and sensitive manner. Many phenolic chemicals have been detected via biosensors based on CC, tyrosinase, polyphenol oxidase and laccase. (Melissa, M. et al., 2015). Among them, laccase has more advantage over tyrosinase and polyphenol oxidase based biosensor.

## 5. Applications:

**Aromatic compounds**

The ability of laccase to degrade various aromatic polymer has led to research into their potentialfor bioremediation and other industrial applications.There are many potential applications for laccase in different industrial and technological sectors. Laccase is used in the textile industry for bleaching of denim fabrics, for enhancement of the whiteness in the peroxide, bleaching of cotton. Aromatic compounds are oxidized by laccase to stimulate homo and/or hetero-coupling processes, resulting in a colour pallet of important textile dyes(including phenoxazine and azo dyes).

**Biosensors**

The most common use of laccaseare in the design of biosensors for the detection of phenolic compounds in food and for environmental and medical applications. Laccase biosensor have been used to monitor some toxic and harmful chemicals like phenolic compounds, pesticide residue and hormones due to their catalytic mechanism, high redox potential and specific/special affinity for phenolic substrates. Laccase enzyme have several industrial applications including dye decolourization, detoxification of environmental pollutants and revalorization or waste and waste water.

**Food**

Laccase is used to improvethe quality of drink and for the stabilization of certain perishable products containing plant oils. In food industry, wine and beer stabilization is the main application of laccase. In the bread making process, laccase affix bread or dough-enhancement additives to the bread dough, these results in improved freshness of the bread texture and the improved mechianibility. It is used in fruit juice processing, baking, improvement of food sensory parameters and sugar beet pectin gelatin. Presence of high concentration of phenolic compounds in the wine affects their taste, smell, colour and gustative sensations. So, removal of polyphenols is necessary to inhibit the modifications in wine.

**Brewing industry**

In the brewing industry, haze formation is a major problem during long-term storage of beer. Due to interaction of proanthocyanidins and specific haze-active proteins, haze is formed. To oxidize the phenol in beer, laccases have been used, polyphenol complexes formed are then removed by filtration and many other separation methods. Removal of oxygen occus at the end of beer production process to increases beer storage life. Very recently, laccase is used in the clarification of fruit juice. For improving dough machinability and the softness of end-product laccase is used.

**Pharmaceutical Industry**

Laccase have been used for the synthesis of several products of pharmaceutical industry. The 1$^{st}$ chemical of the pharmaceutical industry that has been prepared using laccase enzyme is antinocin that has been prepared from 4-methyl-3-hydroxyanthranilic acid. This chemical has anticancer properties and acts by preventing the tumour cell from trancribing DNA. In the organic synthesis laccase is used asa biocatalyst for the catalysis of various antibiotics into amino acids for the synthesis of metabolically stable amino acid analogues. It is also used in the synthesis of anti-cancer drugs (such as mitomycin, actinomycin and vinblastine), immunosuppressors (such ascyclosporin A), antibiotics (such ascephalosporins and penicilin X dimer). After coupling of the radical intermediates, the steroid hormone 17-estradiol and the stilbenic phytoalexinn trans-resveratrol are oxidised, resulting in the formation of dimers and oligomers. In the enzymatic derivation of amino acids, such as L-tryptophane, L-phenylalanine or L-lysine.

**Degradation of xenobiotics**

Laccase is used in bioremediation treatment, in the degradation of industrial effluents and xenobiotics, polyacyclic aromatic hydrocarbons($PAH_s$)(including anthracene or benzopyrene) phenols and organophosphorus insecticides because they are can oxidize xenobiotic compounds. Degradation of aromatic compounds are also done by Rhus vernicifera laccase which is immobilized in fiber membranes. Laccase from R. vernicifera is

used in degradation of phenols which is immobilized in the polypropylene membrane that was modified with chromic acid and subsequently activated by ethylenediamine and GLU.

**Biofuels**

In biofuels, laccase is used to remove phenolic compounds that inhibit the fermentation of the sugar present in the hydrolysate of lignocellulosic materials. In textile industry, to improve the whiteness in conventional bleaching of cotton, laccase is used. To catalyze the oxidation of biomass-based materials for the generation of electrical energy enzyme based biological fuels use enzyme. Reduction of oxygen into water in a four electron transfer step without the intermediate formation of hydrogen peroxide at the expense of the oxidation of a variety of mediators. Production of bioethanol is increased by a laccase produced from T. versicolor expressed in Saccharomyces cerevisiae by eliminating phenolic compounds. Recently, detoxification of lignocellulosic hydrolysates is done by the white rot fungus Ganoderma lucidium. Due to their ability to reduce oxygen into water, laccase have been used in the development of biosensors. Laccase based biosensors has been used in many ways including to quantify fungal contamination in grape musts. In biomedicines, laccasehave been used to detect insulin, morphine and codeine.

**Insects**

Laccase is found in the cuticle of many insects species which oxidizes catecholsin the cuticle ti their corresponding quinines which catalyze protein cross-linking reactions. Laccase is involved in cuticle sclerotization (oxidative incorporation of acylolopamine ). Laccase is detected in following insect genera : Anophales, Apis, Bombyx, Calliphora, Diploptera, Drosophilia, Lucilia, Oryctes, Musca, Rhodnius, for detoxification of xenobiotics, sponges utilize laccase as an antimicrobial agent, used for elimination of lignin-derived products from their filtered food.

In insects, 2 main forms of laccase are found : Laccase 1 and Laccase 2. Laccase 1 is involved in cuticle tanning, laccase 2 is involved for T. Castaneum and M. sexta. In M. sexta, Laccase 1 is found in the salivary glands, the midgut, the Malpighian tubules, the fat

body. Theb generation of melanin in the midgut as a defence gainst parasite invasion is another well known biological function in insects.

**Cosmetics**

In the cosmetic sector, for the production of personal care-products. The items which contains laccase were patented for skin lightening, for bleaching and/or dying hair, laacase is used for removal of $H_2$ $O_2$ that can damage hair and irritate the scalp. $H_2O_2$is used as oxidizing agent in the formulation of hair dyes. A hair colour including laccase and butein was recently developed: either a combination of peroxidase with either $H_2O_2$or a $H_2O_2$ generator.

**Pulp and paper**

Degradation of lignin in wood preparation is necessary. Laccase is used for improving the properties of pulp by formation of reactive radicals with ligin or by funtionalizing lignocellulodic fibers. It also aids in pulp brightening, delignification and the elimination of lipophilic extractives that cause pitch deposition in both wood and non-wood paper pulp. The toxic and colored compounds are released by laccase as effulents from various industries and make them non-toxic by various processes such as polymerization and depolymerizartion reacions.

## 6. CONCLUSION

Laccase is an oldest enzyme used for the oxidation of various phenolic compounds. Laccase belongs to the blue coppper oxidase family which catalyse oxidation reaction coupled to four electron reduction of molecular oxygen to water. Laccase is used in the determination of various phenolic compounds such as xenobiotics, polyphenols, Bisphenol A, catechol.by the formation ofvarious type of biosensor we can easily quantify phenolic compounds. As in case of rutin, rutin is oxidised into o-quinones,which is electrochemically reduced back to original form. Formation of biosensor is done by the combination of laccase with an anion bis(trifluoromethylsulfonyl)imide($Tf_2N^-$) associated with three different imisazolium cations and reference electrode. So, by comparing these we can easily find out. Laccase has many applications in the field of pharmaceutical industry, food industry, brewing industry, xenobiotics, biosensors, aromatic compounds, insects, cosmetics and pulp and paper.

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