International Journal of Advanced Engineering Research and Science (IJAERS)
Peer-Reviewed Journal
ISSN: 2349-6495(P) | 2456-1908(O)
Vol-8, Issue-6; Jun, 2021
Journal Home Page Available: https://ijaers.com/
Article DOI:https://dx.doi.org/10.22161/ijaers.86.12

# Synthetic applications of Laccase and its Catalytic Potentials

Ram Sahay

Department of Chemistry Maharana Pratap P. G. College, Jungle Dhusan Gorakhpur, U.P., India

Received: 02 Apr 2021;

Received in revised form:

03 May 2021;

Accepted: 25 May 2021;

Available online: 13 Jun 2021



***Keywords*— Multicopper, Enzymes, Laccase mediator, Biocatalyst, Phenols, Organic solvents.**

***Abstract*—** *Laccases benzenediol: oxygen oxidoreductase (EC1.10.3.2), multicopper containing oxidoreductase enzymes, are able to catalyze the oxidation of various low-molecular weight compounds, specifically, phenols and anilines, while concomitantly reducing molecular oxygen to water. Because of their high stability, selectivity for phenolic substructures, and mild reaction conditions, laccases are attractive for fine chemical synthesis. This manuscript provides a discussion of the recent applications of this interesting enzyme in synthetic chemistry, including laccase and laccase-mediator catalyzed reactions. There for fungal laccases are consider as a perfect green catalysts is a prominent biotechnological applications. Thus laccases find potential applications in bleaching of paper pulp, biofuel cells and organic synthesis. They can perform transformations from the oxidation of functional group to the hetero nuclear coupling product of new antibiotics derivative.*

## I. INTRODUCTION

Societal interest in green chemistry and advances in biotechnology have brought to the forefront the application of enzymes to address many of the challenges of modern synthetic organic chemistry. This multi-faceted challenge is being addressed by an ever-increasing suite of environmentally benign enzymes. Laccases (benzenediol: oxygen oxidoreductase[EC 1.10.3.2] belong to the multicopper oxidase family, along with such different proteins as plant ascorbic oxidase, mammalian ceruloplasmin ferroxidase from *Saccharomyces cerevisiae*, among others [1]. These copper containing enzymes catalyze the oxidation of various substrates with the simultaneous reduction of molecular oxygen to water [2]. *Yoshida* first discovered laccases in 1883 after observing that latex from the Japanese lacquertree (*Rhus vernicifera*) hardened in the presence of air [3]. This makes laccase as one of the oldest enzymes ever described. Since then, laccase activity has been found in plants, some insects [4], and few bacteria [5]. However, most biotechnologically useful laccases (i.e. those with high redox potentials) are of fungi origin. Over 60 fungal strains belonging to *Ascomycetes*, *Deuteromycetes* and especially *Basidiomycetes* show laccase activities. Among the latter group, white rot fungi are the highest producers of laccases but also litter decomposing and ectomycorrhizal fungi secret laccases [6].

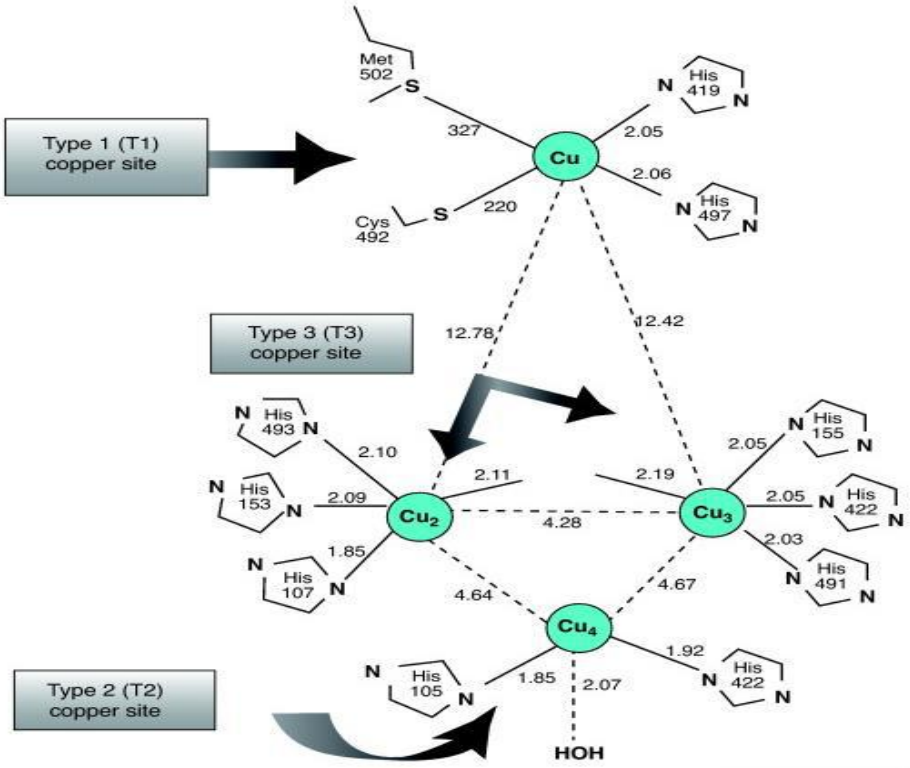


*Fig.1: Scheme of Scheme of T1 (Cu1) and T2/T3 (Cu4/Cu2-Cu3) copper sites of laccase Cot A* from *Bacillus subtilis*, with indicated distances between the most important atoms

Laccases are typically monomeric extracellular enzymes containing four copper atoms bound to 3 redox sites (T1, T2 and T3). The termed blue copper at the T1 site because of its greenish blue colour in its oxidized resting state is responsible of the oxidation of the reducing substrate. The trinuclear cluster (containing one Cu T2 and two Cu T3) is located approx. 12 Å away from the T1 site, and it is the place where molecular oxygen is reduced to water [1] figure-1. Laccases catalyze one electron substrate oxidation coupled to the four electron reduction of $O_2$. It is assumed that laccases operate as a battery, storing electrons from the four individual oxidation reactions of four molecules of substrate, in order to reduce molecular oxygen to two molecules of water. Fungal laccases often occur as multiple isoenzymes expressed under different cultivation conditions. Most are monomeric proteins, although laccases formed by several units have been also described [7, 8]. They are glycoproteins with average molecular mass of 60-70 kDa, and carbohydrate contents of 10-20% which may contribute to the high stability of laccases. The covalently linked carbohydrate moiety of the enzyme is typically formed by mannose, N-acetylglucosamine and galactose. The amino acid chain contains about 520-550 amino acids including a N-terminal secretion peptide [4].

## II. CATALYTIC PROPERTIES OF LACCASES AND MECHANISM OF CATALYSIS

Reduction of dioxygen blue copper-containing oxidases including laccases, there is no general opinion about the electron transfer pathway inside the protein globule and the mechanism of dioxygen reduction in the molecule. The T1 site of laccases is thought to accept electrons from reducing substrates, and then they are transferred onto the three nuclear T2/T3 cluster where molecular oxygen is activated and reduced to water. Interaction of a completely reduced laccase with molecular oxygen results in different forms of the enzyme. Two well-studied forms are termed peroxide intermediate and native intermediate. The native intermediate plays an important role in the catalytic cycle of laccase. During reaction with $^{17}O_2$, this intermediate acts as an oxygen radical shown in figure-2.

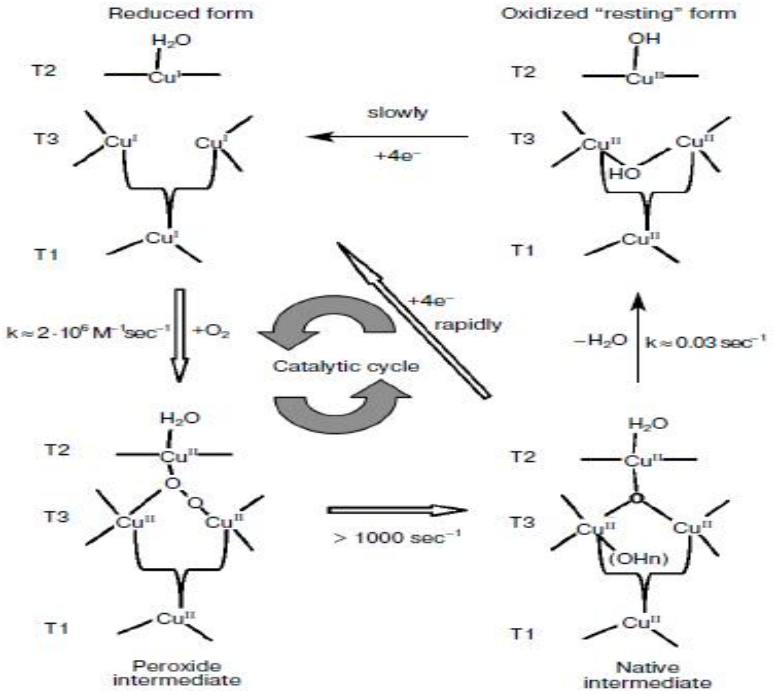


*Fig.2: Catalytic cycle of laccase showing the mechanism of reduction and oxidation of the enzyme copper sites*

## III. BIOLOGICAL FUNCTIONS AND INDUSTRIAL APPLICATIONS

Biological functions attributed to laccases include spore resistance and pigmentation [9, 10] lignification of plant cell walls [11] lignin biodegradation, humus turnover and detoxification processes [8] virulence factors [12] and copper and iron homeostasis [13]. Laccases exhibit an extraordinary natural substrate range (phenols, polyphenols, anilines, aryl diamines, methoxy substituted phenols, hydroxyindols, benzenethiols, inorganic/organic metal compounds and many others) which is the major reason for their attractiveness for dozens of biotechnological applications [14]. Moreover, in the presence of small molecules, known as redox mediators, laccases enhance their substrate specificity. Indeed, laccase oxidizes the mediator and the generated radical oxidizes the substrate by mechanisms different from the enzymatic one, enabling the oxidative transformation of substrates with high redox potentials otherwise not oxidized by the enzyme, figure 1A. The industrial applicability of laccase may therefore be extended by the use of a laccase-mediator system (LMS) figure-2 (A). Thus, laccase and LMS find potential application in delignification and biobleaching of pulp [15] treatment of wastewater from industrial plants [16] enzymatic modification of fibers and dye bleaching in the textile and dye industries [17] enzymatic cross linking of lignin based materials to produce medium density fiberboards [18] detoxification of pollutants and bioremediation [19].

Detoxification of lignocellulose hydrolysates for ethanol production by yeast [20] enzymatic removal of phenolic compounds in beverages wine and beer stabilization, fruit juice processing [21] and construction of biosensors and biofuel cells [22]. In organic synthesis, laccases have been employed for the oxidation of functional groups [23] the coupling of phenols and steroids [24], the construction of carbon-nitrogen bonds [25] and in the synthesis of complex natural products [26] and more. As mentioned above, many of these applications require the use of redox mediators opening a big window for new biotransformation of non natural substrates towards which laccase alone hardly shows activity. On the other hand, in most of the cases large quantities of enzymes are required, which makes the efficient expression of laccase in heterologous systems an important issue. Moreover, the protein engineering of fungal laccases with the aim of improving several enzymatic features (such as activity towards new substrates, stability under harsh operating conditions *e.g.* presence of organic co solvents, extreme pH values-, thermo stability, and others) is a critical point in the successful application of this remarkable biocatalyst. All these issues are addressed in the following lines, paying special attention to their application in organic synthesis.

## IV. LACCASE MEDIATOR SYSTEM

The combination of the laccase with low molecular weight molecules such as 2,2'-azino-bis-(3-ethylbenzothi-azoline-6-sulphonic acid) (ABTS) or 1-hydroxybenzotria-zole (HBT) not only lead to higher rates and yields in the transformation of laccase substrates but also add new oxidative reactions to the laccase repertory towards substrates in which the enzyme alone had no or only marginal activity, figure-2 A, B. Thus, LMS enlarges substrate range being able to oxidize compounds with redox potential (E°) figure2(B) higher than that of laccase (typically, laccase E° at the T1 site is in the range +475 to +790 mV but the LMS allows to oxidize molecules with E° above +1100 mV) [27,28]. Besides, the mediator acts as a diffusible electron carrier enabling the oxidation of high molecular weight biopolymers such as lignin, cellulose or starch [1]. Hence, the steric issues that hinder the direct interaction between enzyme and polymer are overcome by the action of the redox mediator. LMS has resulted highly efficient in many biotechnological and environmental applications as regards the numerous research articles and invention patents published [29]. Many artificial mediators have been widely studied, from ABTS the first described laccase mediator [30], to the use of synthetic mediators of the type-NOH (such as HBT, violuric acid (VIO), N-hydroxyphtalimide (HPI) and N-hydroxyacetanilide (NHA), the stable 2,2,6,6-tetramethyl-1-piperidinyloxy free radical (TEMPO), or the use of phenothiazines and other heterocycles (*e.g.* promazine or 1-nitroso-naphthol-3,6-disulfonic acid) [18] figure-2 (A) . More recently, complexes of transition elements (polyoxometalates) have been also demonstrated to mediate lignin degradation catalyzed by laccase [31]. The choice of a proper mediator (over 100 redox mediators have been described [32] represents a key consideration for a given biotransformation. The use of different mediators may yield different final products when using the same precursors. This is basically due to the fact that substrate oxidation in laccase mediator reactions occurs via different mechanisms. The mediator radicals preferentially perform a specific oxidation reaction based on its chemical structure and effective redox potential (or dissociation bond energy) [33].

Despite all the associated advantages of LMS, there are two major drawbacks hindering the use of mediators: they are expensive and they can generate toxic derivatives. Moreover, in some cases, while oxidizing the mediator, laccase is inactivated by the mediator radicals, or the latter can be transformed into inactive compounds with no more mediating capability (*e.g.* generation of benzotriazol from HBT by losing the hydroxyl group). Last trends are focusing in the use of low-cost and eco-friendly alternative mediators in this sense, several naturally occurring mediators produced by fungi (phenol, aniline, 4-hydroxy benzoic acid and 4-hydroxybenzyl alcohol) have been identified. More recently, phenolic compounds derived from lignin degradation (such as acetosyringone, syringaldehyde, vanillin, acetovanillone, ferulic acid or *p*- coumaric acid) have been demonstrated to be highly efficient laccase mediators of natural origin (even better than the powerful artificial ones) for dye decolorization, removal of polycyclic aromatic hydrocarbons, pulp bleaching and pitch removal [34]. These natural compounds can be obtained at low cost due to their abundance in nature and also in industrial paper pulp wastes, smoothing the progress to a more environmental friendly and sustainable white biotechnology processes figure-3.

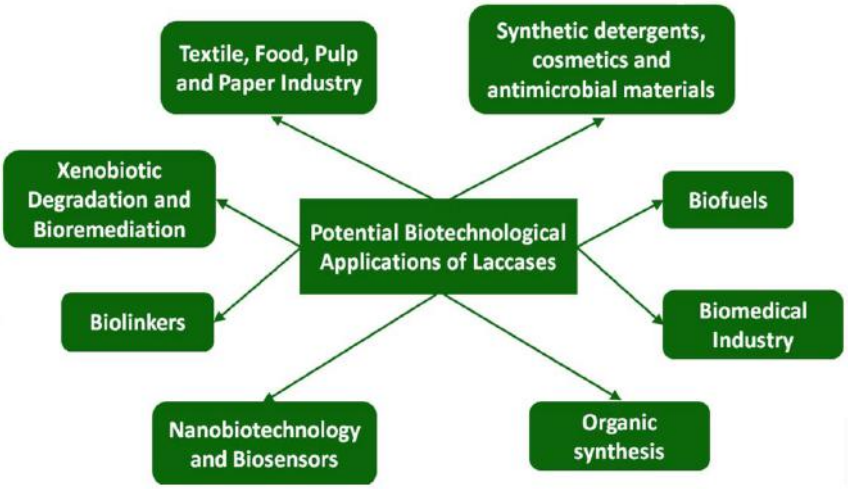


*Fig.3: Potential biotechnological applications of laccase enzyme*

## V. APPLICATIONS OF LACCASES IN ORGANIC SYNTHESIS

Organic synthesis of chemicals suffers from several draw backs, including the high cost of chemicals, cumbersome multi step reactions and toxicity of reagents [2]. Laccases might prove to be very useful in synthetic chemistry, where they have been proposed to be applicable for production of complex polymers and medical agents. Indeed, the application of laccase in organic synthesis has arisen due to its broad substrate range, and the conversion of substrates to unstable free (cation) radicals that may undergo further non-enzymatic reactions such as polymerization or hydration figure-4.

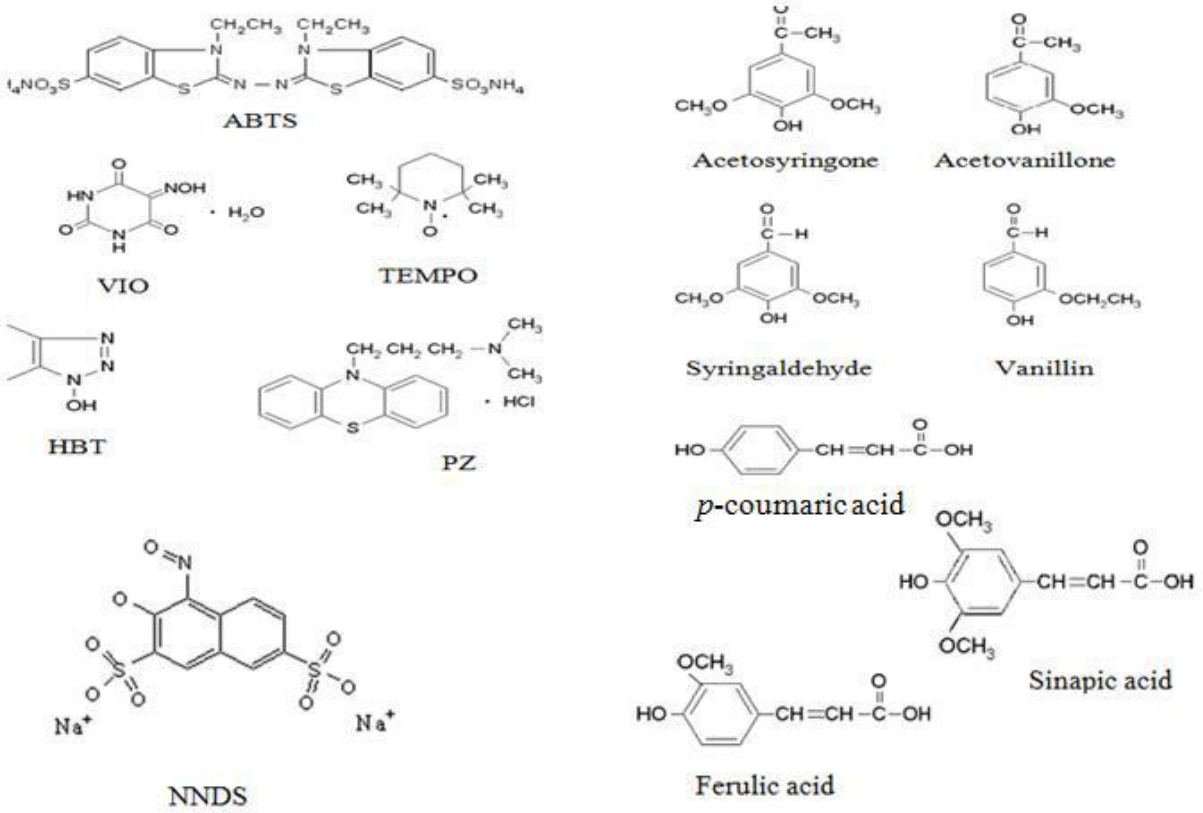


*Fig.4: Structure of some artificial [ABTS, HBT, Violuric acid VIO-, TEMPO, pyromazine, 1-nitroso-naphthol-3, 6-disulfonic acid-NNDS] and lignin derive derived natural mediators [acetosyringone, syringaldehyde, vaniline, acetovanlone, p-coumaric acid, ferulic acid and sinapic acid]*

## VI. LACCASES FOR ENZYMATIC POLYMERIZATION

Enzymatic polymerization using laccases has drawn considerable attention recently since laccase or LMS are capable of generating straight forwardly polymers that are impossible to produce through conventional chemical synthesis [35]. For example, the polymerization ability of laccase has been applied to catechol monomers for the production of polycatechol [36]. Polycatechol is considered a valuable redox polymer; among its applications are included chromatographic resins and the formation of thin films for biosensors. Former methods for the production of polycatechol used soybean peroxidase or horseradish peroxidase (HRP), which suffers from the common suicide $H_2O_2$ inactivation. The main limitation of all heme containing peroxidases is their low operational stability, mostly due to their rapid deactivation by $H_2O_2$ with half lifes in the order of minutes in the presence of 1 mM $H_2O_2$ [37]. Inert phenolic polymers, for example poly (1-napthol), may also be produced by laccase catalyzed reactions [38]. These polymers have application in wood composites, fiber bonding, laminates, foundry resins, abrasives, friction and molding materials, coatings and adhesives [39].

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 [40]. Poly (2,6-dimethyl-1,4-oxy-

phenylene) poly(phenylene oxide), PPO-, is widely used as high-performance engineering plastic, since the polymer has excellent chemical and physico mechanical properties. PPO was first prepared from 2,6-dimethylphenol monomer using a copper/amine catalyst system. 2,6-Dimethylphenol was also polymerized through HRP catalysis to give a polymer consisting of exclusively 1,4- oxyphenylene units [41]. On the other hand, a small amount of Mannich base and 3,5,3'5'-tetramethyl-4,4'- diphenoquinone units are contained in the commercially.

## VII. OXIDATIVE TRANSFORMATION OF ORGANIC COMPOUNDS BY LACCASE ENZYME

Laccases have been used to synthesize products of pharmaceutical importance. The first chemical that comes to mind is actinocin, synthesized via a laccase catalyzed reaction from 4-methyl-3-hydroxyanthranilic acid as shown in figure 7A. This pharmaceutical product has proven effective in the fight against cancer as it blocks transcription of tumor cell DNA [42]. Other examples of the potential application of laccases for organic syntheses include the oxidative coupling of katarantine and vindoline to yield vinblastine. Vinblastine is an important anticancer drug, especially useful in the treatment of leukemia. Vinblastine is a natural product that may be extracted from the plant *Catharanthus roseus* figure-5 and 6.

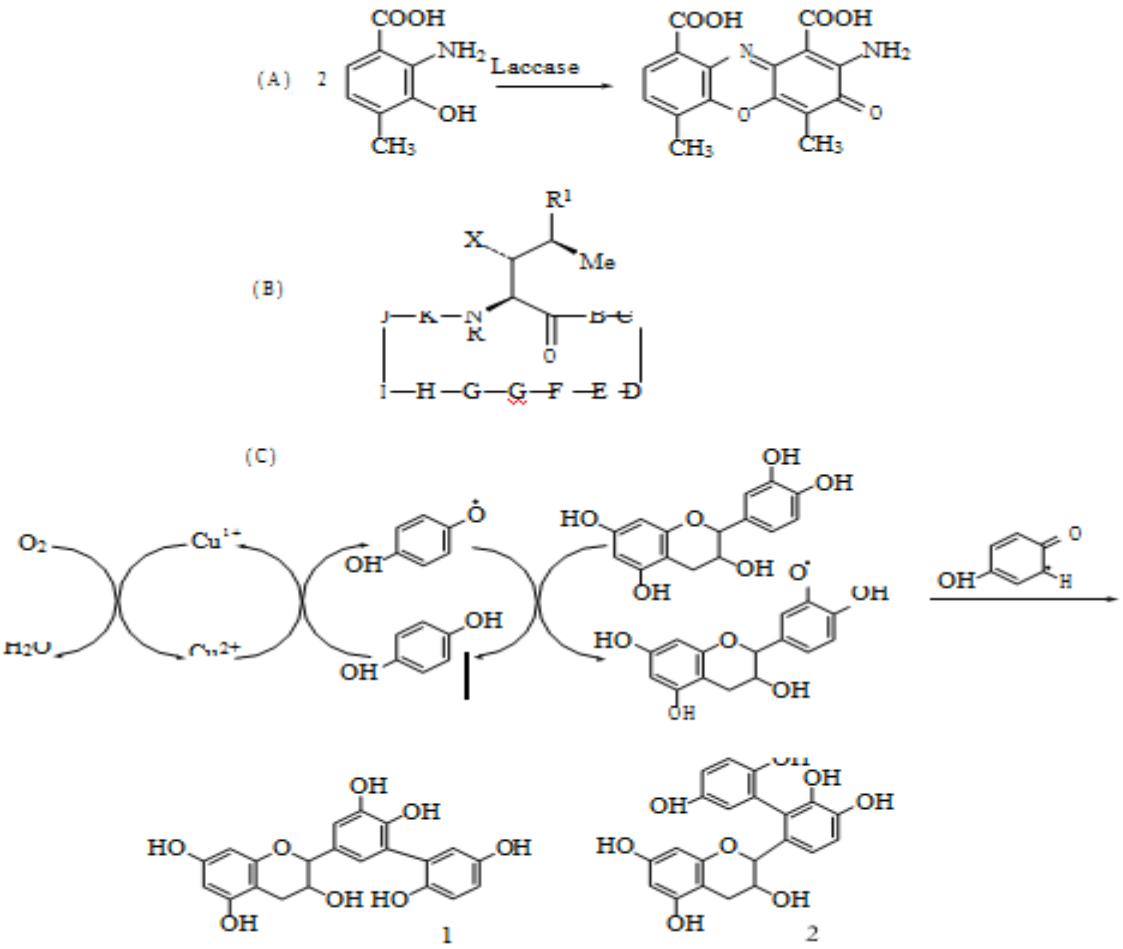


*Fig.5: (A) Synthesis of actinocin via a laccase catalysed reaction, (B) Synthesis of novel cyclosporine reaction product obtained from cyclosporine A by HBT mediated laccase oxidation, (C) Products obtained by the laccase/hydroquinone mediated oxidation of (+) catechin.*

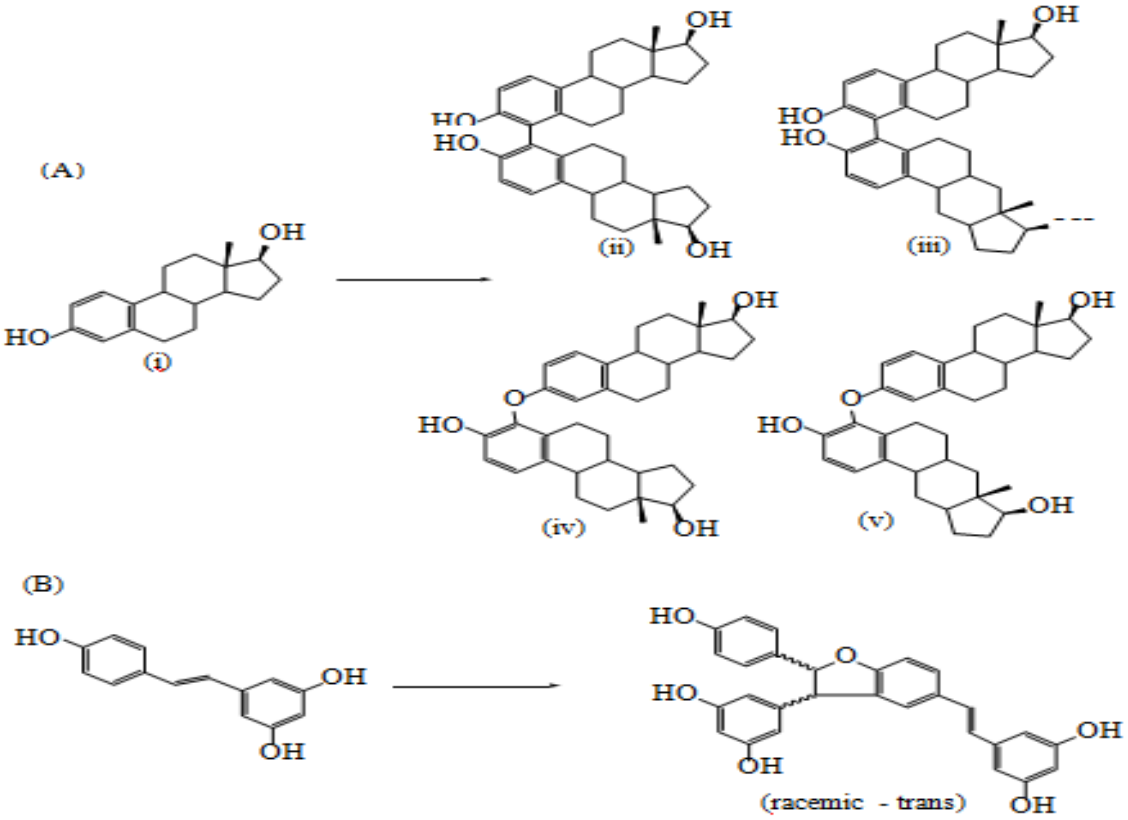


*Fig.6: (A, ii-v) Dimeric products obtained by the oxidationof β-estradiol, (B) Dimeric product obtained by the oxidation of the phytoalexin resveratrol.*

The compound is however only produced in small quantity in the plant, whereas the precursors-namely katarantine and vindoline are at much higher concentrations, and thus are relatively inexpensive to obtain and purify. A method of synthesis has been developed through the use of laccase with preliminary results reaching 40% conversion of the precursors to vinblastine [2] figure 6. Laccase coupling has also resulted in the production of several other novel compounds that exhibit beneficial properties, e.g. antibiotic properties.

## VIII. CONCLUSION

This manuscript demonstrates the usefulness of the laccase in recent synthetic applications. Laccase or laccase mediator systems it provide alternative, environmentally friendly, oxidation methods that can be used to replace a host of traditional chemical oxidants for a wide range of substrates. This increased application of laccase in organic synthesis will future as our understanding of the enzyme structure and mechanism and new laccases are discovered. It is anticipated that the reaction conditions under which laccase performs will be broadened and this will open further research opportunities. In addition, it is also used in fast moving consumer goods (FMCG) as tooth paste, mouthwash, detergent, soap, and diapers in cosmetics as deodorants; in beverage and food industry for wine and juice stabilization.

## ACKNOWLEDGEMENTS

The author Dr. Ram Sahay thankfully acknowledges the support of Department of Chemistry, Maharana Pratap P. G. College Jungle Dhusan Gorakhpur-273014 (U.P.), India.

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