Review of Literature

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REVIEW OF LITERATURE

The review of literature pertaining to the present study entitled **"Optimization of Laccase Production by Isolated Bacterial Sp. using Statistical Experimental Design"** is discussed under the following headings.

2.1 MICROBIAL LACCASES

2.2 PROPERTIES OF LACCASE

2.3 MECHANISM OF CATALYSIS

2.4 LACCASE MEDIATOR SYSTEM

2.5 LACCASE PRODUCTION

2.6 OPTIMISATION OF LACCASE PRODUCTION

2.7 PLACKETT – BURMAN METHOD

2.8 RESPONSE SURFACE METHODOLOGY

2.8 APPLICATIONS OF LACCASE

2.1 MICROBIAL LACCASE

Laccase was first discovered in the exudates of the Japanese lacquer tree *Rhus vernicifera* and subsequently was demonstrated as a fungal enzyme as well Since then, laccase have been found in Ascomycetes, Deutromycetes and Basidiomycetes; being particularly abundant in many white rot fungi that are involved in lignin – metabolism (Kunamneni

et al.,2007) and also laccase activity has also been demonstrated in bacteria like *Azospirillum lipoferum* (Givaudan *et al.*,1993), *Bacillus subtillus* (Martins *et al.*,2002) and a few species of Streptomyces such as *S. cyaneus* ,*S.lavendulae* (Suzuki *et al.,2003)* and *S. coelicolor* (Machczynski *et al.*,2004).

Figure 1
Laccase from *Sterptomyces coelicolor*

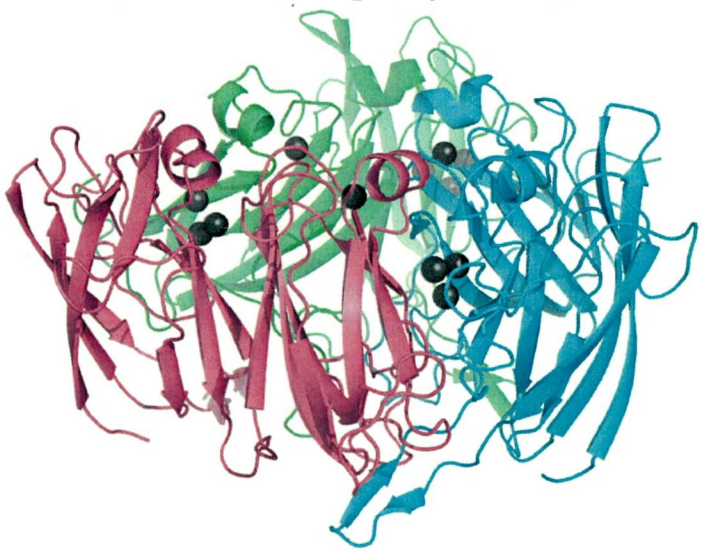
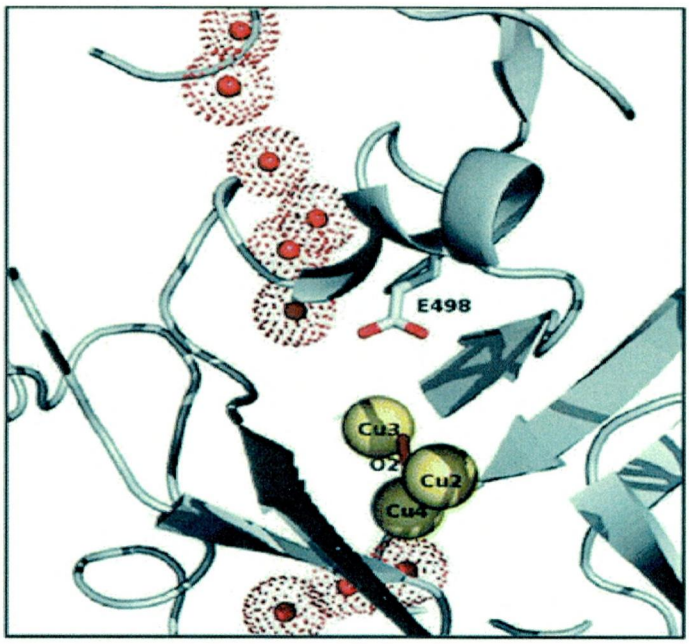


Figure 2

Laccase from *Bacillus Subtillus*



Laccases are the most widely distributed of all the large blue copper containing proteins, as it is found in higher plants and fungi. The fungi producing laccase have been identified by the ability of laccases to oxidise different substrates such as guaiacol, Remmazol brilliant blue (R) (RBBR), tannic acid, poly R-478 and others to specific coloured products (Kiiskinen *et al.*, 2004).

Apart from the usual sources like soil, textile effluents, municipal waste and tree barks, some of the laccases producing fungi belonging to the class Ascomycetes and Basidiomycetes are also isolated from the marine samples. (Verma *et al.,* 2010).

The first bacterial laccase was found in plant associated bacterium *Azospirillum lipoferum*, which was involved in melanin formation. *Azospirillum* bacteria were prevalently found in soil and in the rhizospere of a variety of grasses and cereals. Cultivated plant inoculated with these bacteria shows significant growth improvements. Laccase activity was also reported in a heterocystous cyanobacterium, *Anabaena azollae.* In contrast to fungal laccases, bacterial laccases are highly active and much more stable at high temperature, at high pH as well as high concentrations of chloride and copper ions and the immobilized spore laccases are more compatible with almost all industrial processes (Dwivedi *et al.*, 2011).

Azospirillum lipoferum, where laccase occurs as a multimeric enzyme composed of catalytic subunit and one or two large chains. The enzyme plays role in cell pigmentation and utilization of plant phenolic compounds and transport (Alexander *et al.*, 1999). Other bacteria reported to have laccase are *Streptomyces sp* (Figure 1), *Bacillus subtillus* (Figure 2),

Pseudomonas sp and *Xanthomonas sp.* The molecular weight of bacterial laccase ranges from 50-150 kD (Sharma *et al.,* 2007).

In *Bacillus subtillus* a thermostable cotA laccase, involved in the production of brown spore pigments in endospore coat, was reported. These laccases could help in the protection of spore coat against UV light and hydrogen peroxide (Martins *et al.*, 2002).

Laccase have been isolated from Ascomyceteous, Deuteromyceteous and Basidiomyceteous fungi. In the fungi Ascomycetes and Deuteromycetes have not been a clear focus for lignin degradation studies as much as the white rot Basidiomycetes. The white rot Basidiomycetes are the efficient degraders of lignin (Kunamneni *et al.*, 2007).

Some phenoloxidases showing "Laccase properties" have been purified from larval and adult cuticles of insects such as *Drosophila melanogaster, Lucilia cuprina, Manducta sexta, Sacrophaga bullata* (Gianfreda *et al.*, 2010).

2.2 PROPERTIES OF LACCASE

Most fungal laccases are monomeric, dimeric or tetrameric glycoproteins. Glycosylation of fungal laccase is believed to play a role in secretion, suspectibility to proteolytic degradation, copper retention and thermal stability. Upon purification, laccase enzymes demonstrate considerable heterogenicity, glycosylation content and composition of fungal glycoproteins can vary with growth medium composition. Biochemical characteristics of purified laccase such as molecular weight, optimum pH and temperature, kinetic constants, substrate specificity and effect of inducers, inhibitors and metal ions have been reported in many research works (Desai and Nityanand 2011).

A single organism may also possess several laccase isoenzymes or isoforms, that may differ in their aminoacid sequence and display different kinetic properties towards standard laccase substrates. Fungi produce several isozymes of laccase that differ from one another with respect to both the degree of glycosylation and type of carbohydrate residues. Laccases have been shown to contain four copper(II) atoms per molecule that are essential for its catalytic activity .These four copper(II) atoms can be classified into three groups ,type 1, type 2 and type 3, and are defined in terms of their spectroscopic properties and their electronic potential as determined by their electron paramagnetic absorbance pattern (Ragusa *et al.*,2002).

2.3 MECHANISM OF CATALYSIS

Laccase can catalyze the oxidation of various compounds, including o, p-diphenols, aminophenols, polyphenols, polyamines, lignin, some inorganic ions and aryl diamines. Laccase catalyzes the oxidation of the substrate with concomitant reduction of oxygen to water. It oxidises substrates by removing one electron per time and generates free radicals which can be polymerized (Lu *et al.*, 2007).

Laccases couple the four single electron oxidations of a reducing substrate to the four electron reductive cleavage of the dioxygen bond, using four Cu atoms distributed in to three sites defined according to their spectroscopic properties. Typical metal content of laccases includes one type-1 copper(T1), one type-2 (T2) and two types -3 copper(T3) ions, with T2 and T3 arranged in a tri nuclear cluster (TNC). The type 1 site contains the blue copper, whose tight coordination to a cysteine is responsible for an intense Scys → Cu(II) charge transfer transition at around 600nm,giving the typical blue colour to the enzyme. The T2 shows a characteristics electron

paramagnetic resonance (EPR) spectrum, clearly distinct from that of T1, where as T3 coppers are anti-ferromagnetically coupled and EPR- silent ions.T1 exhibits a planar triangular coordination with the sulphur atom of a cysteine and with the Nδ1 nitrogen of two histidines. The three T2/T3 ions are arranged in a triangular fashion and coordinated to a strongly conserved pattern of four His-X-His motifs. Six of such histidine residues coordinate the T3 copper pair, whereas theT2 is coordinated by the remaining two histidine residues. Electron from the reducing substrate are extracted from the T1, the primary electron acceptor, and then transferred to the TNC through a highly conserved His-Cys-His tripeptide, where the four electron reduction of dioxygen to water takes place. A number of 3D structures of laccases have been solved. All the fungal laccases exhibits a similar molecular architecture organized in three sequentially arranged cupredoxin-like domains. The T1 is located in domain 3, whilst the TNC cluster is embedded between domains 1 and 3 with both domains providing residues for copper coordination (Pisciteli *et al.*, 2010).

The enzymatic oxidation of phenolic compounds and anilines by laccases generate radicals that react with each other to form dimers, oligomers or polymers covalently coupled by C-C, C-O and C-N bonds. In soil, natural and xenobiotic phenolics or aromatic amines can thus be bound to the organic humus matrix. In the case of substituted compounds, the reaction can be accompanied by partial demethylation and dehalogenation (Duran and Esposito, 2000).

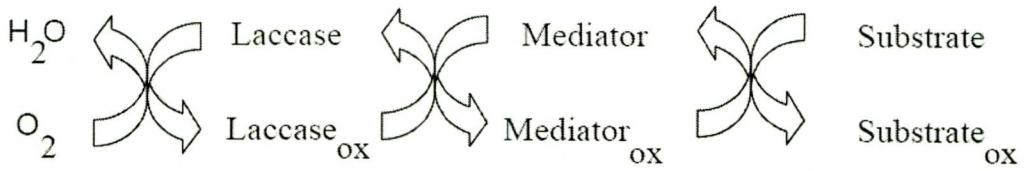
Laccases are involved in the degradation of complex natural polymers, such as lignin or humic acids .The reactive radicals generated, lead to the cleavage of covalent bonds and to the release of monomers. Because of steric hinderance, the enzyme might not come directly into contact with the polymers (Claus *et al.,* 2002).

2.4 LACCASE MEDIATOR SYSTEM

Figure 3

Laccase mediator system

A



B

$$ABTS \xrightarrow[0.7\ V]{Laccase} ABTS^{\cdot+} \xrightarrow[1.1\ V]{Laccase} ABTS^{2+}$$

$$HBT{>}N\text{-}OH \xrightarrow[1.1\ V]{Laccase} HBT{>}N\text{-}O^{\cdot}$$

A mediator is a small redox molecule that acts as an electron carrier between lignin and laccase. The discovery that aromatic mediators could expand the reactivity of laccase towards non-phenolic lignin, has prompted numerous studies in the field of delignification of kraft pulps. Some mediators are 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonate)(ABTS), 1hydroxybenzotriazole (HBT), benzatriazole (BT), remazolbrilliant blue (RBB), chlorpromazine(CPZ), promazine(PZ),1-nitroso-2- naphthol-3-6-disulfonic acid(NNDS) and 4-hydroxy-3-nitroso-1 naphtholenesulfonic acid (HNNS).Mediators ABTS and HBT are those that have been used more in processes such as pulp delignification , decolourisation and detoxification of textile dyes (Octavio *et al.*,2006).

Once the mediator is oxidized, by giving one or several of its electron to laccase, it will diffuse in to the pulp fibre wall to oxidise lignin. The mediator then gains electrons from the lignin fragment that oxidized to

return to its reduced state, making it available once again for oxidation by laccase. Overall, the laccase –mediator system acts as a catalyst to oxidise lignin by transferring electrons from its oxidisable groups to oxygen. The catalytic cycle will go on as there is oxygen present in the system and until oxidation of the lignin becomes too difficult. Contray to laccase, the mediator is able to diffuse within the fibre wall due to its small size and gain better access and therefore to the oxidisable groups of lignin. Also a mediator with high redox potential will likely react more efficiently with phenolic and non-phenolic groups in lignin than a low redox potential mediator, resulting in enhanced delignification (Rochefort *et al* ., 2003).

More than 100 mediator compounds have been described but the most commonly used are the ABTS and the triazole 1-hydroxy benzotriazole (HBT). Various laccases readily oxidise ABTS, by free radicals, to the cation radical ABTS+ and the concentration of the intensely coloured, green-blue cation radicals can be correlated to the enzyme activity (Kunamneni *et al*., 2008).

2.5 LACCASE PRODUCTION

Laccase production has been found to be highly dependent on the conditions for cultivation and nutritive media composition. Laccases were generally produced at low concentrations, but higher yields were achieved with addition of compounds such as xylidine, lignin, veratryl alcohol and ethanol was known to trigger laccase production indirectly (Claus *et al.,* 2002).

Investigation of the effects of different nutrients in the culture media on laccase synthesis is necessary for large scale laccase production (Ding *et al*., 2012).

Laccase is a potentially important industrial enzyme that can be applied extensively in many fields which include waste detoxification and textile dye transformation, delignification of lignocellulosic material and cross – linking of polysaccharides , upgrading wine quality and removal of fermentation inhibitors to increase the yield of ethanol , improvement of drug analysis as well as construction of new energy –producing devices and enzyme sensors(Couto and Herrera, 2006a).

Fungal laccase form an important group of enzymes, as they are involved in the degradation of lignin and in removal of potentially toxic compounds Extracellular laccases are constitutively produced in small amounts in basidiomycetes fungi, But its production can be enhanced considerably by a wide variety of substances such as ferulic acid, 2, 5-Xylidine, p-anisidine or veratryl alcohol as they have structural similarity with lignin or lignin derivatives. Copper plays a key role as metal activator in several fungal laccases cytochrome oxidase, tyrosinases, ascorbic acid oxidase and superoxidase of oxidases group. Copper has a prominent effect on laccase synthesis. Regulation of the synthesis of several laccase isoforms by copper occurs at the level of gene transcription (Madhavi and Lele, 2006).

The production of laccase can be stimulated by the presence of wide variety of inducing substrates, mainly aromatic or phenolic compounds related to lignin or lignin derivatives, such as ferulic acid , 2,5-xylidine ,P-anisidine and veratryl alcohol .Other aromatic compounds that act as inducers include abietic acids ; various phenols (catechol ;4-chlorophenol : 2,6 dimethoxy phenol; guaiacol); several derivatives of benzoic acid (benzoic ,2,6,Dimethoxy benzoic, syringic ,vanillic ,veratric acids);

veratrylaldehyde; the lignin precursors such as coumaric and ferulic acids; lignosulfonates ; and copper sulphate (Pisciteli *et al.*, 2010).

White rot fungi are among the most robust microorganisms, having the ability to degrade the major components of lignocellulosic sources, cellulose, hemicellulose and lignin as available hydrolysable nutrients efficiently through their non-specific and non- stereo selective extracellular enzyme system (Asgher *et al.*, 2011).

Two families of lignolytic enzymes are widely considered to play a vital role in the enzymatic degradation of phenol oxidases and peroxidases. The carbon sources in the medium plays an important role in lignolytic enzymes production. The use of fructose instead of glucose resulted in 100 fold increase in the specific laccase activity of basidiomycetes .Laccase expression in fungi is influenced by the culture conditions such as nature and concentration of carbon and nitrogen sources, media composition, pH, temperature and presence of inducers. The nutritive substances employed in the culture medium constitute significantly to the total production cost (Sivakumar *et al.*, 2010).

Laccase regulation by nitrogen and carbon sources

Laccase activity has also been shown to be dependent on the concentration and nature of carbon and nitrogen sources as well as on their ratio. Nitrogen source plays a key role in laccase production, with effects depending on its nature and concentration in culture media (Pisciteli *et al.*, 2011). Change in laccase activity in response to nitrogen concentration is a controversial issue, since examples of activity increases have been described both limiting and non-limiting conditions. Generally, inorganic nitrogen sources lead to levels of laccase with sufficient biomass production, while

organic nitrogen sources lead to low levels of laccase with sufficient biomass production, while organic nitrogen sources give high laccase yields with good fungal growth. Yeast extract is one of the best nitrogen sources that increases the yield of laccase enzymes (Arora *et al.*, 2002). The enzyme yield is also increased by supplementation of the medium with an additional nitrogen sources like amino acid L-asparagine (Janusz *et al.*, 2007).

As far as carbon sources are concerned, it has been demonstrated that supplementation of substrates, like glucose that are readily utilizable and efficiently metabolized by the microorganism , result in high level of laccase activity. Studies of laccase optimization in *P. ostreatus* IMI 395545 submerged cultures have shown that glucose leads to the highest production of laccase compared to the other carbon sources. As a matter of fact, fivefold increase of the laccase activity has been reported to occur with glucose up to $20gL^{-1}$ (Pisciteli *et al.*, 2011).

Effect of inducers on laccase production

Laccase has broader range of substrates which can induce enzyme production in microorganisms we have determined the capacity of inducers in nine compounds to be suitable for laccase production in each strain. Guaiacol was able to induce laccase in all strains and was used as comparative inducers (Mongkolthanaruk *et al.*, 2012).

Effect of metals on laccase production

The metal ions stimulated laccase formation when added to actively growing culture of *Trametes pubescens* (Galhaup *et al.*,2002).Copper (Cu^{2+}), has been shown to be effective in increasing laccase production in

many organisms at low concentration. The manganese (Mn^{2+}) ion was found to be most effective in stimulating laccase production. Cadmium slightly stimulated laccase production. This implies that the metal ions have effect on enzyme activity more than on enzyme production (Mongkolthanaruk *et al.,* 2012).

2.6 PLACKETT-BURMAN METHOD

Statistical methodologies such as Plackette – Burman Design and Box Behnken design have shown to be efficient approach to systemic investigation on the target factors. PDB is an effective screening design which considerably diminishes the number of experiment and gives information for the evaluation of target factors as possible only the most effective factors with positive significances are selected for further optimization the less significance are selected for further optimization. The less significance or high negative effect on response value would be omitted for further experiments (Placket and Burmann, 1946).

2.7 RESPONSE SURFACE METHODOLOGY

Response surface methodology is a simple model to analyze the effect of various factors influencing the responses by varying them simultaneously. RSM is a very useful tool for this purpose as it provides statistical models which helps in understanding the interaction among the parameters that have been optimized. The advantage of using RSM has been reported to include reduction in number of experimental trials needed to evaluate multiple parameters and the ability of the statistical tool to identify interactions. In addition to analyzing the effects of independent variables, the experimental methodology also generates a mathematical model that describes the overall process (Singh, *et al.,* 2009).

2.8 APPLICATIONS OF LACCASE

Laccases are attractive environmentally friendly enzyme and have shown potential for a variety of applications. Laccases find potential for a variety of applications in pulp delignification and bio beaching in the textile and dye industries treatment of waste water, removal of phenolic compounds in beverages biosensor and biofuel and cell construction and products of pharmaceutical importance. The application of laccases in biotechnological processes requires the production of high amounts of enzyme at low cost, and hence the current focus of research is oriented towards the search for efficient production (Patel *et al.*, 2009).

A number of applications for laccases have been proposed in several industrial sectors, such as textiles, food, paper and pulp, pharmaceutical chemistry, nano biotech, cosmetic, along with their application in bioremediation. As a fact, laccases can be adapted to bleach textiles. As far as bioremediation is concerning laccases may be applied to decolorize textile effluents. To eliminate odour emitted from places such as garbages disposal sites, livestock farms or pulp mills to remove phenolic compounds from olive oil mills and pulp mills waste waters and to decontaminate soils from polycyclic aromatic hydro carbons (Piscitelli *et al.*, 2010).

Textile Industry

Textile dyes now constitute an important sector in the specialty chemicals industry. Unfortunately their high solubility, synthetic origin and diverse and complex molecular structure make their removal a very difficult task (Kudang *et al.*, 2011).

The use of laccases in textile industry is growing very fast, since besides to decolourise textile effluents, laccases are used to bleach textiles,

synthetic dyes and modify the surface of fabrics. The first commercial use of laccases in textile industry was in denim-washing process, where LMS was used to reduce backstaining, enhancing abrasion levels and bleach indigo (Couto and Herrera 2006a).

Bio bleaching

Environmental considerations have led to a search for alternative methods to chlorine bleaching in kraft pulp mills. Biological bleaching has been investigated with fungal lignin degrading enzymes including laccase and manganese peroxidase. Both enzymes have been shown to increase pulp brightness. The use of the laccase mediator system has been receiving increasing attention since the intial discovery that non-phenolic lignin model compounds can be oxidized by laccase in the presence of mediator such as azino-bis(3-ethyl benzo azinoline -6-sulfonic acid)(ABTS) (Annibale *et al.*,2000).

Nanobiotechnology

During the past two decades, bioelectrochemistry has received increased attention. Progress on bioelectrochemistry has been integrated into analytical applications, eg. In biosensors working as detectors in clinical and environmental analysis (Haghighi *et al.,* 2003). Laccases are able to catalyze electron transfer reactions without additional cofactors, their use has also been studied in biosensors to detect various phenolic compounds, oxygen or azides Moreover ,biosensors for detection of morphine and codeine, catecholamines (Fery and leech,2005), plant flavonoids (Wilkolazka *et al.*,2004) and also for alctro immunoassay have been developed (Kuznetsov *et al.*,2001).

Personal care application of laccase

Current hair dyeing or waving processes often involve oxidative or additive chemicals that have unpleasant odours, are irritant to tissues, or are difficult to handle. A laccase –based system may overcome these drawbacks by replacing harsh chemicals and operating at milder condition (in terms of pH and solvents). Laccase catalyzed oxidation, transformation, and cross-linking of various precursors have been reported to result in satisfactory hair dyeing or waving. In addition to providing an easier to handle hair care procedure, a laccase–based system may also improve or complement the cosmetic effect achieved by conventional chemical methods (Kudang *et al.*, 2011).

Many body, domestic and industrial odours are caused by sulfides, thiols, ammonia, amines short chain fatty acids, or other volatile organic compounds. Being able to oxidize various thiols and other sulphur containing compounds, laccases have been studied for deodrants do a laccase system would degrade the offensive molecules or even kill the microbes that generate them. Laccases are also used as a catalyst for the manufacture of anticancer drugs and even as ingredients in cosmetics (Kunamneni *et al.*, 2008).

Food Industry

Laccase can be applied to certain processes that enhance or modify the colour appearance of food or beverage. In this way, an interesting application of laccases involves the elimination of undesirable phenolics, responsible for the browning, haze formation and turbidity development in clear fruit juice, beer and wine. Laccases are currently of interest in baking due to its ability to cross-link biopolymers (Couto and Herrera, 2006b).It was shown by (Selinheimo *et al.*,2006) that a laccase from the white rot

fungus *Trametes hirsuta* increased the maximum resistance of dough and decreased the dough extensibility in both flour and gluten dough.

Pharmaceutical Industry

Laccase-mediated homomolecular and hetero molecular coupling offers an opportunity for the synthesis of new pharmaceutical products, or modification of existing ones, under mild conditions. Laccase –mediated coupling has also been used to synthesis other compounds with potential biological effects such as antioxidative, antitumour, hormonal, anti-inflammatory, analgesic, central stimulant, central depressant, anti-secretory, sedative, anti-proliferative, anti-neoplastic, and 5-lipogenasuppressive effects(Kudanga *et al.*,2011).

Waste Detoxification and Decontamination

Laccase has been used to oxidatively detoxify or remove various aromatic xenobiotics and pollutants found in industrial waste and contaminated soil or water. Laccase catalysis could result in direct degradation or polymerization / immobilization. Reported examples of direct degradation by laccase include direct dechlorination, cleavage of aromatic rings, mineralization of polycyclic aromatichydrocarbons, decolourisation of pulp or cotton mill effluent, and bleaching of textile dyes. The processes include polymerisation among pollutants themselves or copolymerization with other non-toxic substances (such as humic materials). Polymerised pollutants often become insoluble or immobilized, thus facilitating easy removal by such means as absorption, sedimentation or filtration (Xu, 1999).

Biosensor and Diagnostic Application of Laccases

Laccase catalysis (coupled with various physical instruments) could be useful as biosensors for detecting O_2 and a wide variety of reducing

substrates (especially phenols and anilines). Two types of laccase based O_2 sensor are widely used. One type monitors visible spectral changes (at 600nm)of laccases, resulting from the reoxidation of the type I copper(I) in laccase byO_2.Another type monitors current or voltage changes from a modified oxygen electrode on which O_2 reduction is enhanced under the electrocatalysis of immobilized laccase.

For detecting phenols, anilines or other reducing substrates, three types of laccase based sensors have been reported. The first type detects the photometric change resulted from the oxidation of chromogenic substrate, the second type monitors the O_2 concentrations change that is coupled to the substrate, the second type monitors the O_2 concentration change that is coupled to the substrate oxidation, and the third type uses an electrode that replaces O_2 as a electron flown from the substrate (through laccase). For these applications laccase is either immobilized or free in solution, and the coupled physical converter is either optical, amperometric or piezo effect in nature (Madhavi and Lele, 2009).

Potential New Laccase – Based Biocatalyst

Enzymatic catalysis in organic solvents has opened a new field of biotechnological applications of enzymes. The ability to use enzymes in non-aqueous solvents greatly expands the potential scope and economic impact of biocatalysis. When biological catalysts are placed in this unnatural environmental they exhibit a number of remarkable novel properties such as altered stereo-selectivity, enhanced stability and increased rigidity. As well in the presence of organic solvents there is less risk of microbial contamination.

Current and Future Developments

Laccase are promising enzyme to replace the conventional chemical processes of several industries such as the pulp and paper, textile, pharmaceutical and nano biotechnology. However, one of the problems to commercialize the use of laccase is the lack of enzyme stocks. Thus efforts have to be made in order to achieve cheap overproduction of laccase in heterologous hosts, and also their modifications by chemical means or protein engineering, to obtain more robust, active and less expensive enzymes (Camarero *et al.*,2005).