CHAPTER 1

INTRODUCTION

Enzymes have been utilized in several industries for many years. Laccase (EC 1.10.3.2) is one of the important enzymes in terms of applicability and versatility in industries (Dabrimanesh et al., 2015). Laccases are the members of multi-copper oxidases and contain histidine-rich copper binding domains. They can oxidise lignin related compounds and highly recalcitrant environmental pollutants (Brijwani et al., 2010). Moreover, unlike many other oxidoreductases, laccases do not require cofactors such as NAD(P)H and, unlike peroxidases, they do not produce toxic peroxide intermediates. These characteristics of laccases position them as potential industrial oxidative enzymes (Shi et al., 2015).

Laccases are widely distributed in nature and have been described in fungi, plants, and insects and more recently in bacteria and archaea, indicating that the laccase redox process is ubiquitous in nature. Laccase plays an important role in several biometabolic steps, including those involved in fungal pigmentation, plant lignification, lignin biodegradation, humus turnover and cuticle sclerotization, wherein naturally occurring low molecular weight phenolic compounds and natural fiber polymers are utilized as substrates (Jeon et al., 2012). Among the increasing number of bacterial laccases reported several with distinctive functions have been described, including roles in morphogenesis and sporulation process, pigment production and resistance to copper and phenolic compounds (Kim et al., 2015).

Laccases typically comprise of four copper ions of three different types according to spectroscopic properties. Type 1(T1) copper shows an intense electronic absorption band near 610 nm, which is responsible for the blue copper enzyme. Type 2 (T2) copper shows no absorption in the visible spectrum, but is EPR detectable, whereas Type 3 (T3) coppers are antiferromagnetically coupled ions that display an electronic absorption at around 330 nm (Pollegioni et al., 2015). There are some laccases reported not to have a characteristic absorption spectrum.
of T1 Copper. These laccases have been called as yellow or white laccases. Yellow laccases contain four copper ions with an altered oxidized state, due to the integration of putative lignin-derived products in the active site whereas white laccases contain one copper ion, one iron / manganese ion and two zinc ions. These non-blue laccases are present in both fungi and bacteria (Chen et al., 2015).

A remarkable catalytic property of laccase is that it oxidizes various substrates during four-electron transfer process while reducing oxygen to water. Laccase oxidizes several substituted phenols such as guaiacol, veratryl alcohol, dimethoxy phenol and aromatic amines by converting them to the related reactive free radicals, which further undergo depolymerization, repolymerization, demethylation or quinone formation (Mukhopadhyay et al., 2015). The efficacy of oxidation of the substrates generally depends on the type of the substituted group, the position of the substituted group and also the redox potential of laccase (Mogharabi and Faramarzi, 2014).

Laccases have attracted considerable interest in many fields of industry and environmental processes due to their broad substrate specificity and ability to oxidize high redox potential substrates in the presence of certain low molecular weight compounds called as mediators (Diwaniyan et al., 2012). Application of laccase in different industrial fields includes detoxification of industrial effluent, mostly from paper and pulp, textiles and petrochemical industry, used as a tool for medical diagnostics and as a bioremediation agent to clean up herbicide, pesticide and certain explosives in soil. Laccases are also used as cleaning agents in a certain water purification system, as catalyst for the manufacture of anticancer drugs and even as ingredients in cosmetics (Kalia et al., 2014).

Another novel subject of research in the application of laccase is the modification of biopolymers like cellulose and lignocelluloses with a view of altering the properties of paper. Two main approaches used for laccase-assisted modifications are 1) laccase mediated crosslinking of lignin molecules in situ and 2) coupling of low molecular weight compounds into fibers called as biografting (Rencoret et al., 2014).
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The ‘Green chemistry’ approaches aim at the elimination of hazardous substances from chemical processes. The laccase plays a major role in the field of green chemistry or green synthesis as it uses oxygen as oxidant and water as the by-product (Engelmann et al., 2015). In the field of green synthesis, laccase, assisted coupling reactions have potential application in the synthesis of a new pharmaceutical product or modification of existing drug and in beverage processing (Kudanga et al., 2011).

Fungal laccases have been extensively exploited for the application in industrial processes due to their high redox potential. However, till now commercial exploration of fungal laccases is usually hindered due to high fermentation period and low laccase yield (Du et al., 2015). The fungal lacasses required expression in eukaryotic host system and also their applicability is only under mesophilic and acidic reaction conditions (Ricklefs et al., 2014). Since most industrial processes may require harsh conditions such as high temperatures, extremely acidic / alkaline pH and high ionic strengths, fungal laccases usually lose their activities under these conditions (Wang et al., 2015).

In recent years, bacterial laccases or laccase like enzymes have become increasingly prominent. This shift can be attributed to several characteristics of bacterial laccases that may prove beneficial for industrial applications. The biological treatment of industrial wastewater usually requires enzyme to remain active under high pH and temperature or under higher concentration of organic solvent. The bacterial laccases are having highly stable function within a wider pH range and at higher temperatures compared to fungal ones and are also less dependent on metal ions and less susceptible to inhibitory agents (Guan et al., 2015). Laccase from *Thermus thermophilus* having a half-life of inactivation at 80°C of over 14 h and laccase from *Bacillus halodurans* is chloride resistant. In addition, several pH tolerant laccases are described in *Streptomyces* sp. (Santhanam et al., 2011). Bacterial laccases are advantageous for biotechnological applications due to their atypical laccase characteristics, shorter production time spans and ease of genetic manipulation, allowing for better expression in heterologous systems (Prins et al., 2015).
The first bacterial laccase was reported in *Azospirulum lipoferum* in the year 1993. Thereafter laccases have been discovered in a number of bacteria including *Bacillus subtilis*, *Bordetella compestris*, *Caulobacter crescentus*, *E. coli*, *Mycobacterium tuberculosis*, *Pseudomonas syringae*, *Pseudomonas aeruginosa* and *Yersinia pestis* (Dhiman and Shrikot, 2015).

The best studied bacterial multicopper oxidase with laccase activity is CotA from *B. subtilis*, CueO from *E. coli* and SLAC from *Streptomyces* sp. (Sherif et al., 2013). A typical laccase molecule consists of three domains with cupredoxin-like fold probably as the result of gene duplication events. Each domain is formed from a sandwich consisting of seven strands in two beta sheets arranged in Greek-key beta barrel. The T1 centre is located in cupredoxin domain 3 and is coordinated by two nitrogen atoms contributed by histidine residues and sulfur atoms from cysteine residues. The T2 and T3 copper binding centres located between the first and third domain are coordinated by eight histidine residues (four residues in each domain) (Trubitsina et al., 2015).

Along with typical three-domain laccases, bacteria produce two-domain laccases also referred as small laccases. CotA and CueO are three-domain protein, whereas SLAC is a trimer of two-domain protein. Based on the location of their T1 center, two-domain laccases were classified into three different types. Type A contains T1 centre in each cupredoxin domain, whereas single T1 centre present in the second or the first cupredoxin domain of type B and type C (Tischenko et al., 2015). Ausec et al. (2011a) also reported the presence of two-domain and three-domain laccase in the genome of soil samples. All the two-domain laccases showed properties unusual of three-domain laccases in that they are resistant to inhibitor sodium azide and active in the alkaline pH range (Fernandes et al., 2014a).

Most of the bacterial laccases reported are either intracellular or bound to spores. However, in *silico* analysis of bacterial laccase genes reported the presence of extracellular signal sequences and most of the extracellular bacterial laccases are reported in *Streptomyces* sp. (Fernandes et al., 2014b).

Bacterial laccase from native sources are usually not suitable for industrial applications, mainly due to low production yields. However, bacterial systems offer...
more advantageous over fungal systems such as faster multiplication rates, which results in early production (Virk et al., 2012). Laccase production with respect to medium optimization has been extensively studied in fungi, however, there are only a few reports in bacteria. Media optimization is an important and feasible option to reduce cost of production (Singh et al., 2009a). In order to overcome the cost associated with large amount of free laccase required in real application, one of the strategies have been envisaged is the production of the enzyme using cost effective substrates like agrowaste residues (Margot et al., 2013a).

Enzymatic transformation of resistant pollutants is an ecofriendly and promising alternative to the conventional physicochemical methods. Enzymatic treatment is very attractive as it can be accomplished under mild conditions, achieves high reaction specificity and rates and requires relatively small dosage even at industrial scale. When compared with most chemical catalytic processes, enzymatic treatment also consumes less chemicals, water and energy, and produce less waste (Nguyen et al., 2014). The various physicochemical processes like chemical oxidation, solvent extraction and electrochemical method have been limited due to problems such as high cost, low efficiency and generation of toxic-by-products (Asadgol et al., 2014).

Polycyclic aromatic hydrocarbons, bisphenol A, chlorophenol together with other xenobiotics which present in water from several industrial processes are a major source of contamination in soil. These xenobiotics are classified as Trace Organic Contaminants (TOC) which include steroids, hormones, phytoestrogen and other endocrine disrupting compounds, pharmaceutical and personal care by-products and industrial chemicals (Yang et al., 2013).

Adverse toxicological effects of the trace organic contaminants on human health such as inhibited growth of human embryonic cells, reduction in mean birth weight and neurotoxicity have been reported (Alexander et al., 2012). Many of the polycyclic aromatic hydrocarbons may have a detrimental effect on the plants and animals surviving in the areas polluted by these compounds as these compounds are taken up by the plants and in animals they get accumulated via the
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food chain, it can also create severe health hazards and genetic disorders in humans (Sponza and Oztekin, 2010).

Bisphenol A (2,2-bis(4-hydroxyphenyl)propane; BPA), a major component in the production of various consumer products, including plastic packing materials and detergents, is a known endocrine disruptor compound. BPA interferes with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction or behavior (Erkurt, 2015). The role of BPA exposure in the development of prostate and breast cancer, reduction of human sperm counts, alteration of immune function, prevalence of obesity and decreased fertility in fish and mammals has been demonstrated by several studies (Chhaya and Gupte, 2013).

Benzo[a]pyrene (BaP), a polycyclic aromatic compound which contains five fused benzene rings, is widely distributed in terrestrial and aquatic ecosystems due to a variety of anthropogenic activities. Coal-processing waste products, petroleum sludge, asphalt, creosote, and tobacco smoke, all contain high levels of BaP (Bhattacharya et al., 2014). BaP is oxidized by cytochrome oxidase and epoxine hydrolase to give carcinogenic product benzo[a]pyren-7,8-dihydriol-9,10-epoxide which forms adducts with DNA (Verma et al., 2012).

Laccases are reported to effectively degrade a wide variety of trace organic contaminants like pharmaceutical active compounds (Tran et al., 2010), endocrine disrupting compounds (Telke et al., 2009) and polycyclic aromatic hydrocarbon (Zeng et al., 2011). The loss of estrogenic activity of bisphenol A, nonyphenol and octyphenol after laccase treatment was also reported (Catapane et al., 2013). The degraded product of BaP by laccase from Tramates versicolor results in the production of benzo[a]pyrene quinone (Hu et al., 2007).

Keeping in view the importance of laccase in pollution degradation and to increase the tool box of bacterial laccase from different subfamily, bioprospecting new bacterial strains displaying laccase activity are the need of time. Therefore, study on bacterial laccases is important from the perspectives of basic science as well as for the development of novel biotechnological applications.

Hypotheses:
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The study focused on the isolation of thermostable laccase producing bacteria from different environmental samples, optimization of conditions for extracellular laccase production and purification of extracellular laccase. The production of laccase using agrowaste residues was attempted. The purified laccase was investigated for its ability to degrade selected pollutants bisphenol A and benzo[a]pyrene. The following null hypotheses were formulated.

- The bacteria isolated from the selected environmental samples do not produce thermostable laccase.
- The use of selected agrowastes in the culture media does not influence the laccase production
- The purified laccase does not degrade both bisphenol A and benzo[a]pyrene.

In order to test these hypotheses the present research work was designed with the following objectives.

- Isolation and screening of thermostable laccase producing bacteria from different environmental samples.
- Optimization of media components using statistical design of experiments.
- Purification and characterization of laccase.
- Production of laccase using cost effective substrates.
- Application of laccase in the degradation of selected pollutants and analysis of degraded products for toxicity.
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