Chapter 1

Introduction

Laccases (EC 1.10.3.2) are defined in the Enzyme Commission nomenclature as oxidoreductases acting on diphenols and related substances using molecular oxygen as acceptor. They couple the four electron reduction of molecular oxygen to water with the oxidation of a broad range of substrates including phenols, arylamines, anilines, and thiols (Thurston 1994) (Figure 1.1). Furthermore, laccases are also capable of performing polymerization, depolymerization, methylation, and demethylation reactions (Solomon et al. 1996; D’Annibale et al. 2000; Ullah et al. 2000; Held et al. 2005). Because of their wide substrate specificity, laccases have received broad interest for their biotechnological applications in paper pulping, dye decolourization, wood composite production, bioremediation, denim refining, textile cleaning, juice and wine clarification and biosensor and biofuel cell design (Desai and Nityanand 2011), synthesis of natural products like pigments and antioxidants through dimerization of phenolic and nonphenolic acids, manufacture of new compounded material from lignin waste (Huttermann et al. 2001), detoxification of environmental pollutants and revalorization of wastes and wastewaters (Mayer and Staples 2002; Jurado et al. 2009; Pezzella et al. 2015; Senthivelan et al. 2016).

Laccases are widely distributed in all life forms i.e., mammals, plants, fungi, bacteria, archaea, actinomycetes, insects and lichens, with diverse functions. In higher plants it participates in the synthesis of lignin (Sato et al. 2001), whereas, in fungi it has role in lignin degradation, pigment formation, detoxification, free radical scavenging and pathogenesis (Williamson 1994; Eggert et al. 1996; Nosanchuk et al. 2000; Kumar et al. 2015). In bacteria, laccases are found to have roles in melanin synthesis, spore coat resistance, involvement in morphogenesis, and detoxification of copper (Kuznetsov et al. 1984; Roberts et al. 2002; Claus 2003; Sharma et al. 2007). It shows activity towards metal ions like Cu(I) and Fe(II), thus are also classified as metallooxidases (Stoj and Kosman 2005; Brissos et al. 2015). In insects, laccases have been suggested to be active in cuticle sclerotization and catalyze cuticle tanning (Parkinson et al. 2001; Dittmer et al. 2004; Arakane et al. 2005; Hattori et al. 2010).
It has been reported in the venom protein of *Nasonia vitripennis* (Danneels *et al.* 2010), and recently in the malpighian tubules of *Helicoverpa armigera* (Liu *et al.* 2015).

![Reaction mechanism of laccase](image)

**Figure 1.1.** Reaction mechanism of laccase.

The high yield and high redox potential of fungal laccases makes it suitable for various industrial applications. But, the bacterial laccases may have many advantageous properties compared to classical fungal laccases; for example, their highly efficient expression, much higher thermostability (e.g., CotA) (Martins *et al.* 2002), and alkalo-tolerant (such as Lbh1 from *Bacillus halodurans* C-125) nature. Furthermore, existence of intron in fungal laccase genes, formation of disulfide bridges, and glycosylation of fungal laccase are also frequently obstructive. Since spores allow microorganisms to survive under extreme conditions, spore coat laccases were less affected by fungal laccase inhibitors i.e., sodium azide, EDTA (Lu *et al.* 2012), and surfactants like SDS and CTAB (Sondhi *et al.* 2014). Spore laccases which
are active in the alkaline pH range, high temperature and metal chelators could be used for industrial and biotechnological applications (Held et al. 2005), like dye decolourization (Zhang et al. 2013; Loncar et al. 2014; Singh et al. 2015).

Dyes are coloured, aromatic compounds with conjugated system exhibiting resonance of electrons and contain at least one chromophore (colour-bearing group) (Abrahart 1977). Based on their application, dyes are classified as acid, basic, direct, reactive, disperse, sulfur, vat, mordant and azo dyes. Earlier, natural dyes and pigments were used to colour fabrics but after 1856, synthetic dyes were successfully manufactured. The synthetic dyes are color-fast, easy to apply and economic. In spite of so many advantages, the major disadvantage of synthetic dyes is their toxicity, and carcinogenicity, which leads to environmental pollution (Birhanli and Ozmen 2005; Klemola et al. 2007; Verma 2008). Laccases catalyses the degradation or polymerization reaction of the phenolic and aromatic compounds into less toxic by cross-coupling of pollutant phenols with naturally occurring phenols (Huttermann et al. 1980; Jonsson et al. 1998; Ullah et al. 2000). Immobilized laccases are also used for the treatment of phenolic effluents and polycyclic aromatic hydrocarbons (Davis and Burns 1992; Call and Mucke 1997; 1998; D’Annibale et al. 2000). The Bacillus spore laccases are naturally immobilized, thus can be efficiently used in the biosorption and degradation of dyes.

Despite its low redox potential, prokaryotic laccase offer many advantages over eukaryotic laccase. Till date, majority of laccases characterized have been derived from fungi, especially from white-rot basidiomycetes. Further, only a few bacterial laccases have been completely purified and characterized (Koschorreck et al. 2008) but the industrial or biotechnological use of bacterial laccase is still not viable because of its extremely low production yield (Endo et al. 2003). Therefore, it is required to find novel bacterial laccases with potential industrial relevance through the exploration of natural diversity and also to improve the production yield, which would have a tremendous impact on laccase application in a wide array of applications.

Moreover, laccases have also been reported to catalyze the initial steps in melanin biosynthesis from diphenols in Cryptococcus neoformans, thus protecting it from oxidative and microbicidal activities of host cell defense (Williamson and Wakamatsu 1998; Jacobson 2000; Langfelder et al. 2003; Nelson and Lodes 2006; Bliska and
In gram-positive bacteria, MCO, CotA, the endospore coat component of *Bacillus subtilis*, participates in the biosynthesis of melanin-like brown spore pigment responsible for protection against UV light and hydrogen peroxide (Martins *et al.* 2002; Driks 2004; Sharma *et al.* 2007). The multi-copper oxidases (MCOs) are widespread in the members of the family enterobacteriaceae and other pathogenic bacteria with major role in copper homeostasis system in *Campylobacter jejuni* (Hall *et al.* 2008), copper-efflux system in *Escherichia coli* periplasm (Grass and Rensing 2001), copper tolerance in *Vibrio cholera* (Marrero *et al.* 2012) and *Salmonella enterica* Serovar typhimurium (Achard *et al.* 2010), in *Pseudomonas aeruginosa*, the MCOs, ferroxidases, have central role in iron acquisition (Huston *et al.* 2002). In other pathogenic bacteria like, *Staphylococcus aureus*, *Shigella dysenteriae*, *Klebsiella* sp. and *Yersinia enterocolitica*, functions of the MCOs are yet to be discovered (Huston *et al.* 2002; Sitthisak *et al.* 2005; Yang *et al.* 2008; Shao *et al.* 2009).

*Y. enterocolitica* is a gram-negative bacteria belonging to the family enterobacteriaceae. It causes a wide range of intestinal diseases, including enterocolitis with an inflammatory diarrhea, acute terminal ileitis (Kato *et al.* 1977) and mesenteric lymphadenitis mimicking appendicitis (Fredriksson *et al.* 2012). It is also associated with inflammatory bowel disease (Saebó 2005), Crohn’s disease (Persson *et al.* 1975) and other gut related diseases. *Y. enterocolitica* is extremely heterogeneous serologically and is classified into six biovars (1A, 1B, 2, 3, 4 and 5) and more than 50 serotypes (Virdi *et al.* 2012). These are grouped on the basis of pathogenicity into highly pathogenic (biovar 1B), moderately pathogenic (biovars 2-5) and the so called non-pathogenic (biovar 1A) biovars (Mallik and Virdi 2010). Earlier, bioinformatics tools were used to compare the amino acid sequence alignments in *Yersinia pestis* for the four copper-binding domains of MCOs (Kim *et al.* 2001). Recently, Sharma and Kuhad (2009) reported the presence of type 2 and type 3-multi copper oxidase, with copper binding sites, in *Y. enterocolitica*. The bioinformatics analysis has revealed *Y. enterocolitica mco* gene to encode a putative laccase protein (Sharma and Kuhad 2009).

The probable role of laccase in pathogenicity of bacteria is yet to be established, keeping in mind, its well reported role in virulence of *C. neoformans* (Erb-Downward
et al. 2008) and *Salmonella enterica* (Achard et al. 2010). The mutational studies of *Salmonella* in mouse model system shows decrease in its virulence when the multicopper oxidase *cueO* is mutated (Achard et al. 2010). Furthermore, laccase from *C. neoformans* has been reported to oxidize brain catecholamines like L-Dopa and dopamine in highly reactive quinines (Liu et al. 1999) and prostaglandin G2 (PGG2) to produce prostaglandin E2 (PGE2) and 15 keto PGG2, which are identical to their mammalian counter parts (Erb-Downward et al. 2008). When get released in the cytoplasm, they have the ability to form reactive metabolites. Production of O-quinones by laccase can also cause mitochondrial damage and thus can lead to neurodegenerative disorder, like Parkinson disease (PD) (Liu et al. 1999). Laccase from *C. neoformans* catalyses melanin synthesis by oxidizing L-Dopa (Eisenman et al. 2007). This melanin exerts virulence through antioxidative properties (Jacobson and Emery 1991). The antioxidative properties of other fungal laccases (e.g., *Ganoderma lucidum*, *Trametes*) are also well known (Joo et al. 2008; Kumar et al. 2015). The virulence traits expressed by laccase in the pathogenesis of *C. neoformans* have led to the possibility of many other potential roles in enteropathogenic bacteria residing in the human gut (Figure 1.2).

![Figure 1.2. Probable role of laccase in pathogenic bacteria.](image)

Moreover, the laccase gene sequences from *C. neoformans* have been reported to serotypically evolved and distributed and thus used as a novel tool for molecular
Thus the present study was focused on the isolation, cloning, expression and characterization of laccase from non-pathogenic soil bacteria for industrial applications and pathogenic gut bacteria for understanding their evolution and role in pathogenicity.

**Objectives**

Since laccase have several potential applications, the present investigation was planned with the following objectives:

1. Isolation and screening of laccase producing microorganisms.
   - Collection of soil samples from different regions of Haryana.
   - Isolation of bacteria by serial dilution method.
   - Screening of isolates for laccase production.

2. Optimization of laccase production from the selected isolate.
   - Media optimization
   - Effect of different additives

3. Scale up of laccase production at fermentor level.

   - PCR amplification
   - Cloning in pTZ57R/T vector and *E. coli* DH5α host.
   - Sequencing of gene.
   - Cloning and expression in pET28a vector and *E. coli* BL 21 host.
   - Expression and purification optimization.

5. Application of laccase in dye decolorization.