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Production, Purification and Application of Bacterial Laccase: A Review

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Abstract: Laccases are versatile enzymes belonging to the group of oxidases. Laccases catalyzes variety of phenolic compounds, as well as, diamines and aromatic amines with concomitant reduction of molecular oxygen to water. Laccases have mostly been isolated and characterized from plants and fungi in contrast, little is known about bacterial laccases. Thermostability of this particular enzyme makes an attractive feature for their biotechnological application, especially due to its extensive and advanced applications. The applications includes effluents detoxification from the paper and pulp industries, textile industries, petrochemical industries, food, cosmetics, soil bioremediation and biodegradation of environmental phenolic pollutants. The removal of xenobiotic substances and production of polymeric products makes them a useful candidate for bioremediation purposes. In the last few decades, laccase received much attention due to its ability to oxidize both phenolic and non-phenolic compounds. The present review summarizes the distribution of bacterial laccases and their overview in industrial applications in different sectors.

Key words: Bacterial laccase, synthetic dyes, bioremediation, detoxification, xenobiotics

INTRODUCTION

Laccases (benzenediol: oxygen oxidoreduceses; EC 1.10.3.2) are glycoproteins, which are ubiquitous in nature, grouped under blue oxidases in the Enzyme Commission (EC) nomenclature which oxidize diphenols and use molecular oxygen as an electron acceptor (Kiiskinen *et al*., 2004). The laccase was first reported from *Rhus vernicifera*, the Japanese lacquer tree in 1883 (Yoshida) of which the designation laccase was derived and characterized as a metal containing oxidase. This makes it one of the oldest enzymes ever described and has also been detected in insects and bacteria. But the majority of laccases have been characterized from fungi particularly from basidiomycetes, a white-rot fungi which are efficient lignin degraders (Alexandre and Zhulin, 2000). Previous studies have shown that bacterial strains degrade the low molecular weight portions of lignin polymer, unlike fungi which secretes extra cellular enzymes called ligninases. However, due to their productivity, bacterial enzyme systems are expected to serve as useful tools for the conversion of lignin into intermediate metabolites (Gelpeke *et al*., 1999). The aromatic polymer lignin is well known for resistance to microbial degradation because of its high molecular weight and presence of various biologically stable carbon-to-carbon and ether linkages. Microorganisms that degrade plant lignin via oxidative process of fungi, actinomycetes and to a lesser extent, bacteria (Ali and Sreekrishnan, 2001).

To develop an effective, high yielding and economical medium for production of laccase, a suitable substrate specificity and enhanced stability is very important for industrial scale processes. Previous researchers have worked on both solid state and sub-merged fermentation for the utilization of these enzymes. Solid state fermentation mainly utilize the natural lignin materials such as rice bran, wheat bran, coir dust and sugarcane bagasse, etc (Shanmugam *et al*., 2009). But in case of sub-merged fermentation, the media components like synthetic inducers (guaiacol, veratyl alcohol and indulin) were adopted (Kiiskinen *et al*., 2004; Patel *et al*., 2008).

Laccases are applied in many industrial sectors such as paper processing (Couto and Herrera, 2006), discoloration of wine, environmental pollutants detoxification and chemical production from lignin (Win *et al*., 2003). The demand for removal of synthetic dyes from the textile industrial waste using fungal and bacterial laccase is being increased tremendously. Laccase has been reported as an inducible enzyme during degradation of azo dyes by various bacteria (Parshetti *et al*., 2006; Kalyani *et al*., 2008). Investigations on different and less polluting mediators such as natural mediators produced by laccase and also their modification

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by chemical means or protein engineering should be carried out to obtain more robust and active enzymes (Lorenzo *et al*., 2002). This study reviews the distribution, production, purification and applications of laccase in different industrial sectors.

DISTRIBUTION OF BACTERIAL LACCASE

Laccase enzymatic activity is not widely revealed in bacteria. In the last decade, there have been reports indicating laccase-type activity in different bacterial blue multi-copper proteins (Hullo *et al*., 2001; Kim *et al*., 2001). These biocatalysts may have advantageous properties compared to classical laccases; an example is CotA, which has a much higher thermostability than fungal laccases (Martins *et al*., 2002). Claus (2003), stated that only few bacterial laccases have been studied, though rapid progress in genome analysis suggests that these enzymes are also widespread in bacteria.

The most well-known representative is CotA from *Bacillus subtilis*, an endospore coat protein with high thermostability (Hullo *et al*., 2001). Other laccases have been found in *Pseudomonas maltophila* (Isono and Hoshino, 1989), *P. syringae* (copA) (Cha and Cooksey, 1991), *Azospirillum lipoferum* (Givaudan *et al*., 1993), *Xanthomonas campesteris* (Lee *et al*., 1994), *Bacillus* sp. (mnx G) (Van Waasbergen *et al*., 1996), *B. sphaericus* (Claus and Filip, 1997), *P. fluorescens* GB-1 (Okazaki *et al*., 1997), *Aquifex aeolicus* (Deckert *et al*.,1998), *P. putida* GB1 (Brouwers *et al*., 1999), *A. lipoferum* (Diamantidis *et al*., 2000), *P. desmolyticum* NCIM 2112 (Solano *et al*., 2001), *B. subtilis* (Hullo *et al*., 2001), *Pseudomonas* sp. (Francis and Tebo, 2001), *Escherichia coli* CueO (Kim *et al*., 2001; Roberts *et al*., 2002), *P. aerophilum* (pae1888) (Fitz-Gibbon *et al*., 2002), *Oceanobacillus iheyensis* (Takami *et al*., 2002), *a-proteobacterium* SD 21 (Francis and Tebo, 2001), *Streptomycetes* sp. (Arias *et al*., 2003; Endo *et al*., 2003), *c-proteobacterium* JB (Bains *et al*., 2003), *B. halodurans* Lbh-1 (Ruijssenaars and Hartmans, 2004), *Thermus thermophilus* TTC1370 (Miyazaki, 2005) and *Marinomonas mediterranea* (Kalme *et al*., 2007).

PRODUCTION OF LACCASE

Influence of substrates: The processing of agro-industrial waste residues enhance suitable feed stocks for bioconversion into chemicals, including enzymes by fermentation processes, thereby adding value to what normally constitutes a waste product (Giese *et al*., 2008). Laccases catalyze the oxidation of a large variety of reducing phenolic and aromatic compounds, which makes them useful for biotechnological purposes (Couto and Herrera, 2006). These polyphenol oxidases have been obtained from fermentation of agricultural wastes such as tea waste (Muniswaran *et al*., 1994), sago hampas (Smits *et al*., 1996), palm oil waste (Prasertsan *et al*., 1997; Suffian *et al*., 2010), cotton waste (Jaszek *et al*., 1998), corn cob (Couto *et al*., 1999), wheat bran (De Souza *et al*., 2002), banana waste (Reddy *et al*., 2003), barley (Gomez *et al*., 2005), coconut flesh (Couto and Sanroman, 2005), groundnut shell (Couto and Herrera, 2006), sawdust (Vikineswary *et al*., 2006), sugarcane bagasse (Meza *et al*., 2005), banana skin (Osma *et al*., 2007), rice straw (Niladevi *et al*., 2007) and orange bagasse (Giese *et al*., 2008).

Solid State Fermentation (SSF): The production of laccase by bacteria under Solid-State Fermentation (SSF) is found to be economical. It is defined as a process occurrence in absence or near absence of any free-flowing water (Couto and Herrera, 2006). The presence of moisture of about 15% is necessary for solid state fermentation. The most commonly used solid substrates for SSF are cereal grains, wheat bran, sawdust, wood shavings and several other plant and animal materials (Glazer and Nikaido, 2007). The microorganisms grow under SSF conditions are relatively close to their natural habitat (Murugesan *et al*., 2007). There has been an increasing development towards the consumption of organic residues such as agricultural, forestry and industries that produce value added products from raw materials by SSF technique (Kalogeris *et al*., 2003).

In *Streptomyces cyaneus*, a laccase-type phenol oxidase was produced under solid state fermentation conditions and it was suggested that this enzyme was found to be involved in solubilization and mineralization of lignin from a suitable substrate such as wheat straw (Berrocal *et al*., 2000). Vikineswarya *et al*. (2006), produced laccase from *Pycnoporus sanguineus* under Solid State Fermentation (SSF) of sago hampas, Oil Palm Frond Parenchyma tissue (OPFPt) and rubber wood sawdust. Laccase productivity was higher in the range of 7.5-7.6 U g^{-1} substrate during degradation of sago hampas and OPFPt on 11th day of fermentation when compared to rubber wood sawdust (5.7 U g^{-1}).

Sub-Merged Fermentation (SMF): Liquid batch or submerged fermentation (SmF) is frequently used for laccase production with fungi, despite the fact that this mode is quite different from the natural living conditions of these organisms. Fermentation parameters can be controlled easily in liquid batch culture and existing bioreactor configurations have provided satisfactory

laccase production (Thiruchelvam and Ramsay, 2007). Nevertheless, liquid fermentation is not optimal for all fungal species. For example, SmF was more eficient than semi-solid cultivation for laccase production from *T. versicolor* but laccase produced from *T. villosa* under semi solid conditions were found to be favoruable (Minussi *et al*., 2007).

Influence of incubation time and temperature: Thermophilic microorganisms that showed optimal growth temperatures above 45°C have served as a natural source of industrially relevant and thermostable enzymes. The laccases isolated from *Marasmius quercophilus* were found to be optimum at 60°C and also found that pre-incubation of enzymes at 40 and 50°C greatly increased laccase activity (Farnet *et al*., 2000). The best studied bacterial laccase is the cotA gene product (CotA), which is a component of the spore coat of *B. subtilis* (Hullo *et al*., 2001). The important characteristic property of CotA is its thermal stability having a half-life at 80°C of about 2 h and optimum temperature of 75°C (Martins *et al*., 2002).

Similarly, another strain *B. subtilis* produced 1.8 times more laccase on sporulation medium than on non-sporulation medium. Spores oxidized mono- and di-methoxyphenols (0.1 mM) at 50°C (Hirose *et al*., 2003). Koschorreck *et al.* (2008), have cloned a laccase gene (cotA) from *B. licheniformis* and they expressed in *E. coli.* The recombinant CotA protein showed maximum temperature levels at 70 and 80°C, with a residual activity of 43 and 8% during one hour of incubation.

Influence of pH: Many studies have reported a bell shaped pH profile for laccase due to utilization of different substrates and use of molecular oxygen or enzyme itself (Desai and Nityanand, 2011). Murugesan *et al.* (2007) performed an experiment using a typical laccase from *R. praticola* which was found to have an optimal pH at neutral region. Cordi *et al.* (2007) stated that, 3.0-8.0 will be the pH range for laccase when syringaldazine was used as a substrate. Majority of the reports have indicated that initial pH levels were set between pH 4.5-6.0 prior to inoculation but in most cultivation the levels are not maintained (Vasconcelos *et al*., 2000). Laccase produced from *T. modesta* showed an initial pH of 7.0, which is the best for optimal growth and production (Nyanhongo *et al*., 2002).

Influence of carbon and nitrogen sources: The carbon sources in the medium play an important role in ligninolytic enzyme production. The influence of carbohydrates; glucose, fructose, galactose, galacturonic acid, xylose, lactose, sucrose, pectin and inulin, were employed as sole carbon source for the production of laccases by *Botryosphaeria* sp. Veratryl alcohol, a laccase inducer, was added to culture media to study inducible laccase production on the same carbon sources (Elisashvili *et al*., 2002). Jhadav *et al*. (2009) carried out the optimization of laccase production using *Phanerochaete chrysosporium* as source of laccase. Laccase activity was measured using media containing different carbon sources. Laccase production time was standardized using composite minerals such as glucose and guaiacol. The activity was found high in medium containing rice and maize bran than glucose as carbon source.

Ligninolytic systems are activated during the secondary metabolic phase of fungi which are triggered by depletion of nitrogen source (Desai and Nityanand, 2011). Elisashvili *et al.* (2002) observed that ammonium sulphate in the medium increased the laccase activity from *C. unicolor* IBB 62. D'Souza *et al.* (2006) showed glutamic acid and glycine served better organic nitrogen sources than beef extract and corn steep liquor for production of laccase. Although, *S. psammoticus* MTCC 7334 showed yeast extract as the best nitrogen source (34.8 U g^{-1}) and there was no enhancement in enzyme yield with carbon supplementation (Niladevi *et al*., 2007).

Influence of inducers: Laccase production can be considerably stimulated by the presence of inducers (mainly aromatic or phenolic compounds related to lignin or lignin derivatives) such as veratryl alcohol, guaiacol, gallic acid, ferulic acid and ethanol. Also, laccase production can be considerably stimulated in the presence of inducing substances like ethanol, veratryl alcohol, 2,5-xylidine, ferulic acid and guaiacol. Laccase production in γ-proteobacterium JB increased 13-fold due to addition of $CuSO_4$ after the onset of growth. Similarly, Ethidium bromide, Malachite Green, Phenol Red and Thymol Blue have also enhanced the laccase production by 17-, 19-, 4- and 2-fold. They have also isolated an organism from industrial effluent and tested against fourteen aromatic/organic compounds (Kanam *et al*., 2004).

Influence of metal ions: According to Zhang *et al.* (2010), laccase activity was not significantly affected by the presence of Mg^{2+}, Zn^{2+}, Cu^{2+} ions and EDTA at the concentrations of 6.25-50 mM but was reduced by Ca^{2+} at 25-50 mM, Al^{2+} and Fe^{2+} at a concentration of 6.25-50 mM. They have also reported that *Lentinula edodes* laccase was inhibited in the presence of 1 mM Ca^{2+} (70%) and

Zn^{2+} (64%) and was enhanced by 40% in the presence of 10 mM Cu^{2+}. Palmieri *et al.* (2000), reported that the addition of $CuSO_4$ in the production media resulted in 50-fold increase in laccase activity when compared to a basal medium without copper sulphate. Similarly, oxidation of manganese ions played an important role in the function of lignolytic complex of wood degradation, since it efficiently oxidized certain non-phenolic compounds of lignin (Gorbacheva *et al.*, 2009).

Galai *et al.* (2009), identified *Stenotrophomonas maltophilia* AAP56 a soil bacterium by biochemical and molecular methods. The effect of EDTA, sodium azide, urea, Cu^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+} and Ca^{2+} was determined by incubating for 5 min at 4°C prior to substrate addition. The laccase activity recorded 275 U L^{-1} which is increased by 2.6-fold in the production of enzyme. They have also indicated that the enzyme was totally inhibited by the addition of EDTA, which proves it's a metal-dependent enzyme.

Purification of laccase: Laccase produced from *S. psammoticus* was partially purified by ammonium sulphate precipitation and immobilized in alginate beads by entrapment method using calcium and copper. The copper alginate beads proved a better support for laccase immobilization by retaining 61% of the activity when compared to calcium alginate beads which retained 42.5% of laccase activity only (Niladevi and Prema, 2008). Zhang *et al.* (2006) produced laccase from *Panus rudis* under defined shaken liquid culture without induction. The molecular weight of purified laccase enzyme was 58 kDa which contained 8% carbohydrate and an isoelectric point of 3.5. McMahon *et al.* (2007) purified laccase from cell extracts of soil bacterium *P. putida* F6 using a combination of anion exchange chromatography, gel filtration and found increased laccase activity of 747 and 518 U mg^{-1}. The purified laccase has a relative molecular mass of approximately 59 kDa.

Suzuki *et al.* (2003) isolated a laccase from the cell extracts of *Streptomyces lavendulae* REN-7 (STSL). The purified enzyme showed a single protein band on 10% SDS-PAGE with molecular mass of about 73 kDa. Da Cunha *et al.* (2003) determined the laccase activity spectrophotometrically using syringaldazine and observed the absorbance increase during oxidation of substrates under room temperature. According to Jhadav *et al.* (2009), the purified laccase obtained from medium containing glucose and guaiacol showed lower activity than its crude counterpart and the efficiency of purified extract was analyzed by 10% SDS-PAGE. Diamantidis *et al.* (2000) worked on the purification of *Azospirillum lipoferum* laccase by dialysis where the proteins were precipitated from the supernatant with ammonium sulphate. Laccase activity was detected in 30-60% saturated fractions with approximate molecular mass of 60-70 kDa with an acidic isoelectric point (pI) around pH 4.0 (De Souza and Peralta, 2003; Shleev *et al.*, 2004).

APPLICATION OF LACCASE

Pulp and paper industry: In the industrial preparation of paper, the separation and degradation of lignin in wood pulp are conventionally obtained using chlorine or oxygen based chemical oxidants. An enzymatic treatment of non-chlorine bleaching of pulp obtained brighter pulp with low lignin content (Gamelas *et al.*, 2005). Since, wood and other soil materials are naturally degraded by biological origin, the use of lignin degrading enzymes would provide a new alternatives in pulp and paper industries (Madhavi and Lele, 2009). Employing laccase in lignocellulosic fibers will improve the chemical and physical properties of kraft pulp fiber products (Huttermann *et al.*, 2001). Bacterial laccases from *S. cyaneus* CECT 3335 (Arias *et al.*, 2003) and *P. stutzeri* (Kumar *et al.*, 2003) have been examined for bio-bleaching of eucalyptus kraft pulps using ABTS and HOBT (Hydroxybenzotriazole) as redox mediators.

Textile industry: Textile industry effluents cause major pollution problems that contributes two-third of its total dyestuff market which consumes large volumes of water and chemicals during processing of wet cloths (Pearce *et al.*, 2003). Due to their complex synthetic origin, the dye stuff were difficult to decolourize (Zollinger, 2002). Therefore, the development of processes based on laccase found to be immediate solution due to degrading potential towards diverse chemical structure (Salony and Bisaria, 2006).

Campos *et al.* (2001) reported that, purified laccase from *T. hirsute* and *Sclerotium rolfsii* have been able to degrade the indigo dye both in fabric and effluents with the combination of redox mediators. In the recent past, phenol induced laccase from *T. versicolor* was found to be an effective agent for stone washing, denin fabric without using a redox mediators (Pazarloglu *et al.*, 2005). Moreover, Lantto *et al.* (2004) found that laccase with suitable mediator can activate the wool fabrics for the anti-shrink treatment. Basto *et al.* (2006) proposed the use of ultrasound treatment for efficient cotton bleaching by laccase. Mustafa *et al.* (2005) used hydro-organic medium that produced stable yellow coloured products by means of oxidation of ferulic acid by laccase, which can be easily recovered.

Bioremediation: The involvement of laccase in biodegradation was mainly due to its catalytic properties (Maciel *et al*., 2010). Laccase are used for decolorizing dye house effluents that are hardly decolourized by conventional sewage treatment plants (Novotny *et al*., 2004). The xenobiotic compound present in contaminated soil, Polycyclic Aromatic Hydrocarbons (PAHs) in natural oil deposits and fossil fuels were easily degraded by laccase (Pointing, 2001; Anastasi *et al*., 2009). Laccase was found to be responsible for the transformation of 2, 4, 6-trichlorophenol to 2, 6-dichloro-1, 4-hydroquinol and 2, 6-dichloro-1, 4-benzoquinone. Laccase Mediator System (LMS) have been also used to oxidize alkenes, carbazole, ethylcarbazole, fluorene and dibenzothiophene (Niku-Paavola and Viikari, 2000). LMS has been extensively study for the oxidation of recalcitrant PAHs and several other contaminants (Alcalde *et al*., 2006).

Food industry: Laccase substrates such as carbohydrates, unsaturated fatty acids, phenols and thiol-containing proteins are important components of various foods and beverages (Kirk *et al*., 2002). Various enzymatic treatments have been proposed for fruit juice stabilization, among which laccases are one of the choice of treatment (Alper and Acar, 2004). Minussi *et al*. (2002) have reported the potential application of laccase in different forms such as beverage processing, bioremediation (food industry waste water), ascorbic acid determination, pectin gelation, baking and also act as biosensor. Several studies reported the use of laccase in ascorbic acid determination, sugar beet pectin gelation, baking and as well as olive mill wastewater treatment (Couto and Herrera, 2006; Selinheimo *et al*., 2006; Minussi *et al*., 2007).

Other sectors: In organic synthesis, laccase become a new biocatalyst that extended its application during the last decade (Mayer and Staples, 2002). Laccase acts as a mediator in the polymer production process without enzyme catalyzed cross-linking (Ikeda *et al*., 2001). Moreover, *T. versicolor* laccase expressed in *S. cerevisiae* have improved the production of fuel ethanol from renewable raw materials (Larsson *et al*., 2001).

Many products generated by laccases are antimicrobial, detoxifying or active personal care agents, anesthetics, anti-inflammatory, antibiotics and sedatives (Nicotra *et al*., 2004). Due to their specificity and bio-based nature, laccase become a field of interest in pharmaceutical sector. Laccase have shown capable of fighting against aceruloplasminemia disease (a medical condition of lacking ceruloplasmin, a multi-Cu serum oxidase whose ferroxidase activity regulates iron homeostasis) (Harris *et al*., 2004).

CONCLUSION

Bacterial laccases are overcoming the disadvantages of instability and in-process applications when compared to fungal laccase. They are highly active and much more stable at high temperatures and high-pH values. Bacterial laccases become an industrially important enzyme that are applied in various processes like detoxification of industrial effluents, mostly from the paper and pulp, textile and petrochemical industries, important tool for medical diagnostics, cleaning agent for certain water purification system and catalyst for manufacturing anti-cancer drugs. The important obstacles to commercialize the bacterial laccases was the lack of sufficient enzyme stocks and the cost of redox mediators. Thus, efforts have to be made in order to achieve cheap over-production of this biocatalyst and also alteration of enzyme by chemical means to obtain more robust and active enzymes.

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