Appl Microbiol Biotechnol (2015) 99:155–164
DOI 10.1007/s00253-014-6219-0

MINI-REVIEW

Critical factors affecting laccase-mediated biobleaching of pulp in paper industry

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Received: 16 August 2014 / Revised: 6 November 2014 / Accepted: 7 November 2014 / Published online: 26 November 2014

Abstract Next to xylanases, laccases from fungi and alkali-tolerant bacteria are the most important biocatalysts that can be employed for eco-friendly biobleaching of hard and soft wood pulps in the paper industry. Laccases offer a potential alternative to conventional, environmental-polluting chlorine and chlorine-based bleaching and has no reductive effect on the final yield of pulp as compared to hemicellulases (xylanases and mannanases). In the last decade, reports on biobleaching with laccases are based on laboratory observations only. There are several critical challenges before this enzyme can be implemented for pulp bleaching at the industrial scale. This review discusses significant factors like redox potential, laccase mediator system (LMS)—synthetic or natural, pH, temperature, stability of enzyme, unwanted grafting reactions of laccase, and cost-intensive production at large scale which constitute a great hitch for the successful implementation of laccases at industrial level.

Keywords Laccase · Biobleaching · Redox potential · Grafting · pH · Mediators

Introduction

Laccases are multicopper oxidases found in fungi, bacteria, plants, and insects; they can oxidize a wide array of organic and inorganic substrates in the mild as well as extreme environmental conditions. Due to their clean reaction mechanism having only water as byproduct, they are very attractive as industrial biocatalysts. Laccases contain three types of copper atoms that can be distinguished by UV/visible and electronic paramagnetic resonance (EPR) spectroscopy. On catalytic sites, the type 1 copper catalyzes the electron transfer, type 2 activates the molecular oxygen, and type 3 is responsible for oxygen uptake. Enzymes lacking the Cu atom responsible for the blue color are called "yellow" or "white" laccases, but widely considered, they are not true laccases (Yaropolov et al. 1994; Morozova et al. 2007; Shleev et al. 2007). Due to continuous criticism and pressure rising by several environmental protection agencies to cease or reduce the direct or indirect involvement of chlorine or chlorine-based chemicals in pulp and paper industries, research efforts have been accelerated worldwide for replacing chemical pulp bleaching technologies with green technologies or bio-based alternatives. One of them is to uphold discoveries of novel pulp delignification enzymes produced by fungi and bacteria. Generally, enzyme-based delignification of pulps occurs below 100 °C and atmospheric pressure. But their proteineous natures, like all enzymes, are proteins that are often sensitive to extreme pH and temperature which influences their three-dimensional structure (Call and Mucke 1997). These biocatalysts cause no deteriorating impact on ground water reservoirs and no risk of soil pollution (Singh et al. 2007, 2011a, b; Virk et al. 2012; Niels and Kepp 2013). The potential of giving enzymatic treatments to pulp was realized in the mid-1980s, when it was discovered that xylanase pretreatments of pulps prior to their subsequent bleaching with chlorine, chlorine dioxide, and hydrogen peroxide could yield substantial reduction in bleaching chemicals (Viikari et al. 1986). Later on, use of lignin degrading enzymes, exclusively the laccases, has been shown to be more effective than xylanases at laboratory or pilot scale studies. Inclusion of laccase before chemical bleaching could reduce the use of chlorine-based chemicals in paper industries (Singh et al. 2011a, b; Virk et al. 2012). Less use of bleaching chemicals

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means less need of fresh water to wash chemicals from the bleached pulp. The paper industry consumes about 300 m^3 of water per ton of paper produced and generates a huge volume of highly colored and toxic carcinogenic effluents (Buzzini and Pires 2007). Initially, the use of laccase for bleaching of hard wood pulps had limited acceptance due to minimal delignification that could be achieved. This inefficiency was attributed to less redox potential and size of the enzyme made it unable to diffuse into pulp fibers to catalyze the oxidation of lignin. Fortunately, this problem was circumvented when Bourbonnais and Paice (1990) discovered that laccase in presence of 2,2 -azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) could delignify the kraft pulp. Since then, various research groups have been focusing their attention in search of cost-effective laccase mediator (synthetic or natural) system (LMS) for increasing redox potential of enzyme. Except for a very few alkali-tolerant laccases (Singh et al. 2008; Eugenio et al. 2011), the rest of the biobleaching studies with these enzymes are based on acidic or neutral pH (3.0–6.0). Pulp and paper industries look for highly alkali-tolerant laccases for delignification purposes, because several steps of paper making pass through the alkaline environmental conditions (Singh et al. 2008, 2011a, b). The next hurdle for laccases before bleaching at industrial scale is that they need high oxygen pressure for efficient functioning. On the other hand, majority of laccases have less specific activities; as a result, excessive enzyme units are required before implementing at large-scale biobleaching projects. Less thermo stability of laccases is also a discouraging factor for successful implementation of enzyme at industrial level. Recently, grafting of biobleached pulp was observed by many workers when reaction mixture contained natural mediator and laccase. Although grafting provides strength to pulp fibers but increases the kappa number (KN) and reducing the brightness. Search for the cost-effective methods of enzyme production has always been an important area of enzyme technology. Conventional methods of enzyme production are not appropriate for the large-scale production of laccases from fungi or prokaryotes. Less production of enzyme is a big challenge to continuous supply of laccases to pulp and paper industries at large scale. This review is the first representation of consistent difficulties in the path of successful implementation of laccases at industrial scale for eco-friendly biobleaching of pulps.

Low redox potential (E0) of laccases, inappropriate for proper delignification of pulp

Copper-containing oxidases can be divided into three groups: high, medium, and low redox potential enzymes. Low potential laccases have E0 below 430 mV, medium potential ones have 470–710 mV, and higher potential means an E0 above 710 mV (Morozova et al. 2007). Laccases can only oxidize those compounds whose ionization potential (energy required for removing an electron from its atom) do not exceed the redox potential of T1 copper center. Laccase substrates include aromatic compounds. Many of these compounds are phenolic fragments of lignin. The main purpose of laccases in nature is lignin polymerization and depolymerization (Arora and Sharma 2010). It is believed that for effective delignification of pulp, laccase should be cleaving the non-phenolic lignin structures that depend on the redox potential of the T1 center. The redox potential of non-phenolic lignin structures, such as veratryl alcohol, 1,2-dimethoxybenzene and others are high (>1400 mV). However, it has been demonstrated that laccase is able to oxidize some compounds (redox mediators) with a higher redox potential than laccase itself, although the mechanism by which this happens is unclear. In the presence of such redox mediators, laccase is able to oxidize non-phenolic lignin model compounds and decrease pulp kappa number to a great extent. The standard redox potential range for laccase activity is usually between 500–800 mV versus normal hydrogen electrode (NHE) range which can attack only phenolic moieties comprising less than 10 % of the total polymer in natural lignin (Martínez et al. 2009). Plentiful non-phenolic units in lignin, have redox potential above 1300 mV. The reported redox potentials of laccases are lower than those of non-phenolic compounds (Table 1), so these enzymes cannot oxidize such rigid substances. This limits the range of substrates that can be oxidized by laccases and consequently constrains the delignification of pulp. The E0 of mediators play negligible role in the catalytic efficiency of lignin oxidation; their effectiveness is likely to depend on the chemical reactivity of the radical formed after their initial step of oxidation (Arzola 2009). The possibility to expand their range of oxidation through redox mediators offers considerable biotechnological potential for biobleaching of pulps. The use of such compounds has particular merit because once they are oxidized by laccases to stable radicals, these radicals may continue oxidizing other compounds, including those not used directly as substrates of the enzyme.

Dependency of laccases on mediators for delignification of pulp is cost intensive

Some laccases have higher molecular weight and cannot penetrate deep into the pulp fibers; moreover, due to their rather low redox potential, they are unable to oxidize non-phenolic lignin units. Due to of these limitations, laccase alone can oxidize only phenolic lignin units (<20 % of lignin) on the substrate surface. Therefore, laccases are often applied with an oxidant mediator for biobleaching purposes. A mediator could be a small molecule that acts as an “electron shuttle” between enzyme and substrate. Since the use of ABTS as a mediator

Table 1 Redox potential ($E°$) of laccases from different sources

Name of laccase producing source	Redox potential (mV)	Taxonomic identification of organisms	Reference
Pycnoporus cinnabarinus	810	Basidiomycetes	Sigoillot et al. 2004
Trametes ochracea	790	Basidiomycetes	Shleev et al. 2004
Trametes versicolor	780	Basidiomycetes	Reinhammar 1972
Trametes hirsuta	780	Basidiomycetes	Kojima et al. 1990
Trametes villosa	780	Basidiomycetes	Xu et al. 1996
Cerrena maxima	750	Basidiomycetes	Shleev et al. 2004
Pycnoporus sanguineus BRFM 66	746	Basidiomycetes	Uzan et al. 2009
Marasmius quercophilus C30 (*I*)	730	Basidiomycetes	Klonowska et al. 2002
Pycnoporus sanguineus BRFM 902	729	Basidiomycetes	Uzan et al. 2009
Pycnoporus coccineus	723	Basidiomycetes	Uzan et al. 2009
Trichoderma harzianum	692	Ascomycetes	Sadhasivam et al. 2008
Basidiomycetes C30	560	Basidiomycetes	Klonowska et al. 2002
Coprinus cinereus	550	Basidiomycetes	Schneider et al. 1999
Scytalidium thermophilum	510	Ascomycetes	Berka et al. 1997
Myceliophthora thermophila	470	Basidiomycetes	Xu et al. 1996
Rhus vernicifera	430	Magnoliophyta	Reinhammar 1972
Rhus vernicifera	410	Magnoliophyta	Johnson et al. 2003

for biobleaching of pulp is considered as cost intensive (Singh et al. 2011a, b) at the industrial scale and hydroxybenzotriazole (HBT) has also been shown to inactivate laccases after some time and possesses high toxicity even at low concentrations (Ibarra et al. 2006; Medina et al. 2013), therefore, an extensive search is required to find out cost effective and non toxic natural mediators (Camarero et al. 2007; Reiss et al. 2013). It has been reported that the treatment of different kinds of pulps with Lignozyme—laccase mediator system (LMS) could reduce the kappa number by 70 %. Lignozyme introduced a new mediator, N-hydroxyacetinalide (NHAA) that is biodegradable and has been claimed to be cost effective. Biobleaching with NHAA allows the laccase to maintain about 80 % of its activity after 1-h treatment, but HBT causes a severe loss of enzyme activity. A delignification of >40 % can be obtained with most of the pulps by using 5 kg t^{-1} HBT, in many cases a dosage of 2.5 kg t^{-1} HBT pulp was sufficient (Call and Mucke 1995, 1997). Actually, natural mediators are phenols which can be easily extracted from pulping liquors (Camarero et al. 2007; Gutiérrez et al. 2007), effluent streams (Ismail et al. 2005), and plant materials. Natural mediators have been found to perform similarly or even better than synthetic mediators, with increased activity and more modest useful rates (Canas and Camarero 2010). They do not cause inactivation of the enzyme and represent the promising alternative for environmentally friendly delignification of pulps. The first evidence of natural phenols (syringaldehyde (SA) and acetosyringone (AS)) to mediate delignification of eucalypt pulp by laccase from *Pycnoporus cinnabarinus* at pH 4.0 and 50 °C (Camarero et al. 2007). When sinapic acid, ferulic acid, coniferyl aldehyde, and sinapyl aldehyde were evaluated as laccase mediators, they lead to lower bleaching efficiency for sisal pulp as these phenolic compounds tend to bind to pulp fibers (Aracri et al. 2009). Fillat et al. (2010) evaluated SA, AS, and p-coumaric acid (PCA) as natural mediators for laccase from *P. cinnabarinus* at pH 4.0, 50 °C to bleach flax fibers. Efficiency of these three was compared to HBT in terms of laccase stability. HBT and PCA were found to inactivate laccase in absence of pulp. All natural mediators resulted in a reduced KN after the subsequent alkaline treatment with hydrogen peroxide. Generally, natural mediators increased KN, decreased brightness, and changed optical properties of the pulp after the L stage, suggesting that natural mediators tend to couple to fibers during a laccase mediator treatment (Andreu and Vidal 2011). Therefore, the search for new and more effective mediators involved in natural lignin degradation is needed. In addition, from the point of view of industrial and environmental application, laccase mediators should be environmental-friendly and available at low cost.

Grafting effect of natural mediators on pulp fibers is disappointing for biobleaching

During laccase-based biobleaching of pulps in the presence of natural mediators, the delignification process is hindered by adverse reactions, and then it's called grafting. The phenoxy

derivatives besides lignin oxidation react in several different ways (Fig. 1). Although some authors have emphasized that laccase-catalyzed grafting of phenols onto lignocellulose fibers is an advantageous phenomena, because of their interest in this area, like production of lignin-rich fibers (Aracri et al. 2009). Natural phenols polymerize to yield oligomers or undergo grafting reactions via radical coupling to the pulp surface. Increase in the KN after grafting makes subsequent alkaline hydrogen peroxide extraction mandatory for increasing the brightness of pulp. Barneto et al. (2012) investigated the structural relationship of various natural phenols in biobleaching of kenaf and sisal pulp. It was found that grafting increased the KN of pulp. The mediator that yielded least stable radicals (p-coumaric acid) was the best to graft onto pulp fibers. Most reactive radicals (lacking methoxy groups at ortho-position) show the greatest tendency to collapse with lignin and the least tendency to collapse with other mediator molecules (polymerization). It was concluded that phenolic mediators bearing methoxy groups in ortho-positions show the lowest tendency to undergo grafting reactions and the highest to undergo polymerization. These reactions compete with delignification, representing an adverse and undesirable phenomenon in biobleaching process. At mill scale, biobleaching with natural mediators will not be considered as cost-effective, until an extra step of washing with H_2O_2 or NaOH is added for increasing the brightness of pulp.

Acidic working pH of laccases is not appreciable for biobleaching at industrial scale

Pulping is an initial process of paper making, which involves cooking of lignin containing raw material in the presence of NaOH at high temperature and pressure (pH >12 and cooking times 0.5–3 h at temperature 160–180 °C). During pulping, excess of the lignin is removed from the pulp, but still, some embedded intact lignin needs later chemical or enzyme bleaching. After pulping, the pH of pulp is around 9–12 and needs washing with water for reduction in pH. Inclusion of acidic laccases for delignification after pulping stage will add an extra step of reducing pH of pulp; as a result, increase in time and cost of the biobleaching process. Therefore, isolation of novel alkali tolerant laccase for application in biobleaching of pulp is required. Most of the fungal laccases have acidic (3.6 to 5.2) pH optima, while plant laccases works best between 6.8 and 7.4 pH (Dwivedi et al. 2011; Pezzella et al. 2013). Some bacterial laccases showed alkaline pH optima for biobleaching of pulp and decolorization of dyes (Singh et al. 2008; Virk et al. 2013). The pH optima of laccases depend on the nature of substrate (Singh et al. 2007). This variation may be due to changes in the reaction caused by the substrate, oxygen, or the enzyme itself (Kunamneni et al. 2007). *Streptomyces cyaneus* CECT3335 laccase was reported for biobleaching of eucalyptus kraft pulp at pH 5.0 with ABTS as mediator (Arias et al. 2003). Aracri et al. (2009) delignified sisal pulp at pH 4.0 using laccase from *Trametes villosa* by trying different mediators (viz. sinapic acid, ferulic acid, coniferyl aldehyde, and sinapyl aldehyde). Eugenio et al. (2010), optimized pH 3.0 for the biobleaching of kraft pulp by using laccase from *Pycnoporus sanguineus* with acetosyringone as a mediator. Laccase from γ-proteobacterium JB was the first report on biobleaching of agro-based wheat straw-rich soda pulp with alkaline pH 8.0, ABTS as a mediator. The organism grew well from pH 6 to 10 and produced laccase maximally at pH 10 (Bains et al. 2003; Singh et al. 2007, 2008, 2009a). Optimum pH 10 was reported for the modification of alkaline lignin by laccase from entophytic fungus, *Mycelia sterilia* YY-5 (Weihua and Hongzhang 2008). Moreover, laccases have an advantage over other lignin oxidizing enzymes, like lignin peroxidases (Lip). Redox potential of Lip increases with decrease in pH of reaction environment. For laccases, such data is not available; as logically, alkali-tolerant laccases will be the best choice for pulp bleaching where as alkaline conditions are demanded at large (Singh et al. 2009b; Canas and Camarero 2010).

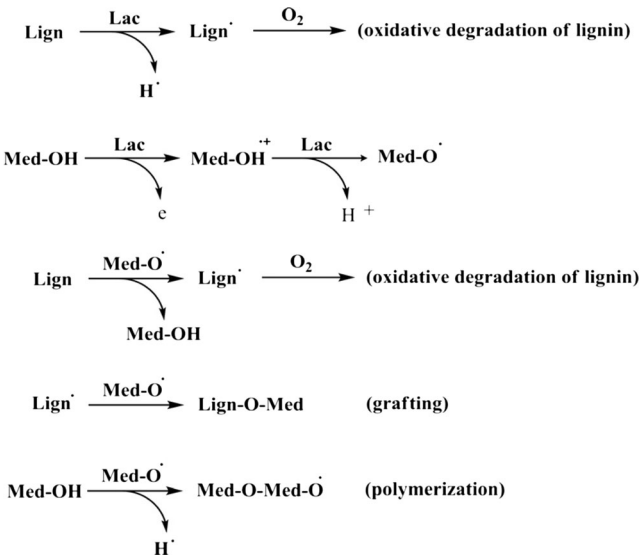

Fig. 1 Reaction mechanism of natural phenolic mediators and laccase as a result appearance of grafting of pulp fibers (Barneto et al. 2012)

Low thermostability of laccases is not a favorable factor for biobleaching of pulps

Thermostability of enzymes is an attractive feature for their biotechnological applications (Berka et al. 1997; Singh et al. 2010, 2011a). Laccases are viewed as moderately thermostable and considerable emphasis has been directed for the isolation of increasingly thermostable varieties. Kiiskinen et al. (2004) isolated novel laccases from wood

rotting fungi, most of them had $t^{1/2}$ at 60 °C of 3–6 h, but pH optima were 2.0–4.0. Reiss et al. (2011) reported laccase from *Bacillus pumilus* that lost 50 % activity after 1 h at 65 °C, pH (5–7). Laccase from the spores of *Bacillus vallismortis* retained more than 50 % activity after 10 h at 70 °C and demonstrated broad pH stability in both acidic and alkaline conditions (Zhang et al. 2013). Weihua and Hongzhang (2008) reported laccase activity was enhanced with increasing reaction temperature with syringaldazine and reached a maximum at 60 °C, but thereafter, it dropped rapidly at higher temperature. The fungal laccases generally have lower thermal stability than bacterial laccases (Hildén et al. 2007; Singh et al. 2011a, b). More thermostable laccases can reduce the reaction time for biobleaching at industrial scale.

Specific activity of laccases and enzyme dosage are crucial aspects for biobleaching

The most striking characteristic of enzymes is their specific activity, that increases as the biocatalyst preparation becomes more pure, since the amount of protein (mg) is typically less, but the rate of reaction remains the same or more due to less interference or removal of inhibitors. Pulp and paper industries are ambitious for high-performance laccases having high specific activity which in turn will be beneficial, because lower enzyme dose will be required for delignification of pulp. Several researchers have optimized the laccase dose in order to use the least amount of enzyme for maximum biobleaching of raw pulp. Studies have revealed that optimum enzyme dose as 10–20 Ug^{-1} pulp for the laccase-mediated biobleaching (Arias et al. 2003; Camarero et al. 2004; Singh et al. 2008; Aracri et al. 2009; Fillat et al. 2010; Babot et al. 2011). However, for laccase from *Streptomyces*, an enzyme dose of 2.4 Ug^{-1} pulp was considered optimum for the delignification of kraft pulp in the presence of acetosyringone as mediator (Eugenio et al. 2011). According to recent studies (Dedhia et al. 2014), 22 Ug^{-1} pulp laccase dose was optimum for delignification of wheat straw pulp at pH 3.5, 60 °C with HBT as a mediator. Table 2, shows that laccase dose optimized by majority of researchers for biobleaching of pulp is between 10–20 Ug^{-1} of pulp. This indicates that for biobleaching of 1.0 ton of pulp, industry will need of 10 to 20 millions of laccase units. Requirement of such huge units of laccases can be reduced by dedicated efforts to isolate such microbes that can produce laccases with high specific activity.

Presence of oxygen is an essential factor for biobleaching of pulp

Laccases need O_2 to oxidize the lignin components embedded in pulp, by the reduction of one O_2 molecule to give one molecule of water plus the oxidized form of the substrate (Yaropolov et al. 1994; Madhavi and Lele 2009; Eugenio et al. 2011). Bourbonnais and Paice (1996) reported ~32 % delignification (after alkaline extraction stage) when kraft pulp was reacted with laccase from *Trametes versicolor* in the presence of ABTS under 100–400 KPa of O_2 for 2 h. Moldes et al. (2008) reported that O_2 pressure was a significant factor for the biobleaching of eucalyptus kraft pulp with laccase from *Trametes villosa* at pH 4.0, 50 °C. O_2 pressure of 600 KPa brought 3.0 to 4.0 % improvement in brightness in all bleaching sequences. Balakshin et al. (2001) have found that most dioxygen takes part in side reactions, even after laccase has lost all activity and only marginally in delignification reactions. The improved pulp properties obtained at a high O_2 pressure may have resulted from the delignifying effect of O_2 itself. Fillat and Roncero (2009) reported that, the presence of increased amounts of O_2 (~4.0–7.0 ppm) during laccase mediator treatment resulted in more efficient oxidation of lignin of flax pulp and hence, decreased in KN. The presence of increased amount of O_2 in the medium enhances the ability of LMS to modify the embedded lignin in pulp to a greater extent than it increases the ability of the enzyme treatment to remove the polymer.

Recombinant laccases in biobleaching of pulps

There is keen research and development (R&D) interests for bringing advancement in application of laccases in the pulp and paper industry. This is demonstrated by a high number of published scientific papers and patents on this topic that continued to increase every year, since 1995. On the other hand, development of laccases for pulp and paper sector is greatly concentrated by biotech companies like Novo Nordisk and Novozymes. Data from the annual report demonstrated that this company has been consistently increasing the investments in R&D and increasing its sales in enzymes for the pulp and paper industry (Demuner et al. 2011). The catalytic improvements of laccases for the better pulp delignification have been tried by the use of some modern biotechnological techniques such as recombinant DNA, mutagenesis, PCR, and encoding. The *Aspergillus oryzae* laccase producing strain was developed by transformation of *A. oryzae* host strain How B711 (derived from the A 1560 strain) with two plasmids pRaMB17.WT and pToC90. The pRaMB17.WT plasmid contains the laccase gene from a thermophilic fungus *Myceliophthora thermophila* that occurs in decaying manure and other organic matter. The laccase gene is linked to the DNA regulatory sequences, promoter, and terminator. The laccase preparation is marketed under a trade name "Flavourstar." Sigoillot et al. (2004) investigated the pulp bleaching efficiency of *P. cinnabarinus* laccase expressed in two *Aspergillus* hosts (*A. oryzae and Aspergillus niger*), and

Table 2 Laccases from fungi and bacteria, optimized conditions (physical and chemicals) for biobleaching of pulp

Laccase producing organism	Laccase Ug^{-1} pulp	Reaction time (h)	pH of process	Reaction temp. (°C)	Redox mediator used and (concentration mM or %)	Type of pulp/ consistency (%)	Results and outcome of the study	References
Trametes versicolor (ATCC 20869)	5.0	2.0	5.0	60	ABTS (1*)	Spruce kraft, mixed softwood kraft, mixed hardwood kraft, and sulfite pulp/10	Laccase–ABTS treatment delignify first three pulps and sulphite pulp up to 40 and 50 %, respectively	Bourbonnais and Paice (1996)
Trametes versicolor	5.0	2.0	5.0	60	ABTS and HBT (10**)	Softwood kraft pulp/10	HBT showed more delignification and less residual laccase activity as comparison to ABTS, 37, 2.0, and 34, 32 % respectively	Bourbonnais and Paice (1996)
Coriolus versicolor	10	8.0	4.5	40	HBT and N-hydroxyacetanilide (NHAA)/0.1	Pine kraft/10	NHAA showed very fast delignification at the beginning of the process as a result of fast formation of the oxidized mediator species	Balakshin et al. (2001)
Streptomyces cyaneus CECT 3335	10	3.0	5.0	45	ABTS (5)	Eucalyptus kraft/10	Reduction in the kappa number by 2.3 U and increased in brightness by 2.2 %	Arias et al. (2003)
1. Laccase from *Pycnoporus cinnabarinus* (wild strain) 2. Recombinant laccases were produced in *Aspergillus oryzae* and *A. niger* hosts by *lacI* gene of wild strain	NA	NA	5.0	NA	HBT/NA	Wheat straw Kraft pulp/10	The laccase expressed in *A. niger* has the same efficiency in delignification as the wild-type laccase (close to 50 % compared with the control trial without laccase), whereas the laccase expressed in *A. oryzae* showed no delignifying effect	Sigoillot et al. (2004)
Pycnoporus cinnabarinus	20	12	4.0	50	HBT (3*)	Eucalyptus globulus/3.0	Laccase activity was inhibited 50 and 20 % in presence of HBT and pulp + HBT, respectively within 4 h. Laccase–HBT promote delignification (four points-decrease of kappa number) and 6 % increase brightness.	Ibarra et al. (2006)
Trametes villosa	17	2.0	4.0	50	HBT (1.5*)	Eucalyptus kraft/10	The laccase–HBT treatment brought delignification (by 20–27 % decrease in kappa number)	Moldes et al. (2008)
Aspergillus fumigatus VkJ2.4.5	10	2.0	6.0	50	HBT (1.5*)	Mixed wood/10	Kappa number decreased by 14 and brightness improved by 7 %	Vivekanand et al. (2008)
γ-proteobacterium JB	20	4.0	8.0	55	ABTS (2)	Wheat straw/10	Enhanced brightness by 5.89 and reduced kappa number by 21.1 %	Singh et al. (2008)
Trametes versicolor	20	2.0	4.0	45	Violuric acid (74)	Eucalyptus globulus and *Pinus pinaster* kraft pulp/2.5	Reduction in kappa number and increase in brightness of *E. globulus* and softwood pulp by 49 and 10 % and 35.9 and 11 %, respectively	Oudia et al. (2008)
Trametes villosa	20	4.0	4.0	50	Sinapic acid, ferulic acid, coniferyl aldehyde, and sinapylaldehyde HBT (1.5*)	Sisal (alkaline pulp from soda–anthraquinone cooking process were)/ 5.0	HBT inactivated the laccase by 99 and 78 % in absence and presence of pulp, respectively. Natural mediators proved less efficient than HBT in facilitating	Aracri et al. (2009)

Table 2 (continued)

Laccase producing organism	Laccase Ug^{-1} pulp	Reaction time (h)	pH of process	Reaction temp. (°C)	Redox mediator used and (concentration mM or %)	Type of pulp/ consistency (%)	Results and outcome of the study	References
							pulp bleaching; rather, they tended to bind to pulp fibers.	
Pycnoporus cinnabarinus	20	5.0	4.0	50	Acetosyringone, syringaldehyde and p-coumaric acid which were compared in performance with (HBT). (1.5 or 3*)	Flax/3.0	All natural mediators reduced the kappa number after subsequent alkaline treatment with hydrogen peroxide. HBT and p-coumaric acid were inactivated the laccase in the absence of pulp.	Fillat et al. (2010)
Trametes villosa	17	2.0	4.0	50	HBT and violuric acid and natural mediator syringaldehyde (SyAl) (1.5*)	Eucalyptus kraft/10	HBT and violuric acid gave high elignification and brightness values (similar to industrial TCF pulp) where as SyAl improved pulp properties in a lower extent.	Moldes et al. (2010)
Pycnoporus sanguineus	2.4	1.0	3.0	40	Acetosyringone (0.05)	Eucalyptus kraft/10	Lowers hydrogen peroxide consumption down to 87.4 % (94.0 % without L) and enhances brightness up to a 59 % ISO (51 % ISO without L).	Eugenio et al. (2010)
Commercially available enzyme (laccase gene from *Myceliophthora thermophila* expressed in *Aspergillus oryzae*) Novozyme 51003	22	10	3.5	60	HBT (1.5*)	Wheat straw/5.0	In the presence of HBT laccase was inhibited up to 75 % after 12 h.	Dedhia et al. (2014)

L treatment with laccase, *NA* information not available from cited article, *HBT* hydroxybenzotriazole

*Stand for %

**Stands for mediators used in mg/g pulp

comparing with the native enzyme. Laccase from wild *P. cinnabarinus* and *A. niger* with HBT as redox mediator achieved a delignification up to 75 %, whereas the recombinant laccase from *A. oryzae* was not able to delignify the pulp. Three laccases were different and each fungal strain introduced differences during protein processing (folding and/or glycosylation). In order to improve laccase treatments of pulp, Ravalson et al. (2009) synthesized a chimeric laccase by fusing *P. cinnabarinus* laccase *lac1* to the carbohydrate-binding module (CBM) of *A. niger* cellobiohydrolase B. The chimeric protein was investigated for its softwood kraft pulp biobleaching potential in comparison with the native counterpart. By conferring to the chimeric protein the ability to bind to a cellulosic substrate, CBM addition greatly improved laccase delignification properties. The recombinant laccase from *Thermus thermophilus* was applied to the biobleaching of wheat straw pulp. Optimized conditions for biobleaching were 3.0 U laccase g^{-1} dry pulp at 90 °C, pH 4.5, 8 % consistency for 1.5 h. Pulp brightness was increased by 3.3 % ISO, and the pulp kappa number was decreased by 5.6 U. Pulp biobleaching in the presence of 5 mM ABTS further increased the pulp brightness by 1.5 % ISO (Zheng et al. 2012).

Cost-effective production is a challenge for continuous supply of laccase to pulp and paper industries

Madhavi and Lele (2006) reported the highest (692 U ml^{-1}) laccase production from newly isolated fungus WR-1, in the presence of 0.8 mM 2,5-xylidine as an inducer in optimized growth medium. Niladevi et al. (2007) suggested that response surface methodology (RSM) could be used as a valuable statistical method for the optimization of enzyme production from *Streptomyces psammoticus*; but using this strategy, only threefold enzyme productions was enhanced in comparison to conventional method of enzyme production. Singh et al. (2008) reported the biobleaching of wheat straw-rich soda pulp by alkali-tolerant laccase from γ-proteobacterium JB. Production of this laccase was low (8×10^3 nkat L^{-1}) with conventional method of enzyme production (OVAT system). Later, RSM-based optimization of enzyme production increased laccase production up to 9.3-fold (Singh et al. 2009c). There are several reports of laccase production enhancements available in literature from fungi, but still favorable results not forthcoming according to the pulp and paper industries requirements. Mate et al. (2010) carried out the directed evolution of a laccase gene from basidiomycete PM1, expressed in *Saccharomyces cerevisiae*. After replacing the native signal sequence with α-factor prepro–leader to regulate the heterologous protein, the fusion protein was subjected to eight rounds of laboratory evolution in combination with rational approaches. The last mutant OB1 showed 34,000-fold enhancement in laccase activity.

Conclusion

Although, several reports are available on laccase-based biobleaching of pulps, still much remains to be discovered and learned about these biocatalysts (Singh et al. 2011a, b; Virk et al. 2013), like clarity about chemistry and mode of action of LMS for efficient and cost-effective delignification of pulps. There is a need to isolate such organisms which can produce alkalophilic, thermostable laccases, possessing high redox potential and specific activity. Sincere efforts are needed to overcome the limitations like low production and cost of mediators that hamper the industrial implementation of laccases.

Acknowledgments Authors are thankful to SERB/DST, Delhi, India, for providing the research funding under fast track young scientist program (SB/FT/LS-315/2012).

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