Thermo and halo tolerant laccase from *Bacillus* sp. SS4: Evaluation for its industrial usefulness

(Received April 8, 2018; Accepted April 13, 2018; J-STAGE Advance publication date: June 28, 2018)

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Abstract

Laccases are unable to oxidize the non-phenolic components of complex lignin polymer due to their less redox potential (E0). Catalytic efficiency of laccases relies on the mediators that potentiates their oxidative strength; for breaking the recalcitrant lignin. Laccase from *Bacillus* sp. SS4 was evaluated for its compatibility with natural and synthetic mediators (2 mM). It was found that acetosyringone, vanillin, orcinol and veratraldehyde have no adverse effect on the laccase activity up to 3 h. Syringaldehyde, p-coumaric acid, ferulic acid and hydroquinone reduced the enzyme activity ≥50% after 1.0 h, but laccase activity remained 100 to ~120 % in the presence of synthetic mediators HBT (1-Hydroxylbenzotrizole) and ABTS (2, 2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) after 3 h. MgSO₄ and MnSO₄ (40 mM) increased the enzyme activity 3.5 fold and the enzyme possessed ≥70 % activity at a very high concentration (2 M) of NaCl. The enzyme retained 40-110% activity in the presence of 10% DMSO (dimethylsulfoxide), acetone, methanol and ethyl acetate. On the other hand, CuSO₄ (100 µM)
induced the laccase production 8.5 fold without increasing the growth of bacterial cells. Laccase from SS4 appropriately decolorized the indigo carmine (50 µM) completely in the presence of acetosyringone (100 µM) within 10 min and 25 % decolorization was observed after 4 h without any mediator.

**Key words:** *Bacillus* sp. SS4, laccase, mediators, redox potential (E0), thermo and halo tolerant

**Introduction**

Laccases from prokaryotes have received the wide attention of researchers because of their ability to act as robust industrial biocatalysts at mild to higher temperatures, acidic to alkaline pH (Miyazaki, 2005; Singh et al. 2007; Sondhi et al. 2014; Singh et al. 2015) and at a higher salt concentration (Singh et al. 2008; Liu and Hua, 2014). Since the first report came on laccase-producing bacterium *Azospirillum lipoferum* (Givaudan et al. 1993) and later delignification of Kraft and agro based pulp under acidic and alkaline conditions by bacterial laccases (Arias et al. 2003; Singh et al. 2008), the enhanced delignification of woody biomass (steam pretreated poplar) by small laccase (sLac) from *Amycolatopsis* sp. 75iv3 (Singh et al. 2017) created a huge interest in prokaryotic laccases. Despite the several functional advantages of bacterial laccases over their counterpart fungi, they cannot perform absolutely in the absence of small-molecular weight compounds called redox mediators that act as an electron shuttle between the enzyme and substrate to form stable radicals, and oxidizes even non-specific substrates of laccases (Bourbonnais and Paice, 1990; Camarero et al. 2007; Singh et al. 2008). Virk et al. (2013) reported a laccase from *Rheinheimera tangshanensis* that can de-ink old newsprint without any mediator in catalytic reaction, which indicated that bacterial laccases have possibilities and abilities that can be exploited in the paper and textile industry without the use of cost intensive mediators. It is, thus, imperative to isolate diverse bacterial laccases and to identify their potential applicability to oxidize a wide range of substrates, especially non-phenolic units of
lignin. Although, in recent years, extensive isolation of prokaryotic laccases has been attempted, and a few bacterial laccases have been characterized, however in general, these enzymes are not suited to the harsh industrial conditions in the pulp and paper industries (Singh et al. 2015). Most bacterial laccases are intracellular or spore bound, and this restricts their implementation on an industrial scale by making the downstream processing expensive (Sharma et al. 2007; Gupta et al. 2017). *Bacillus* sp. SS4 (Gene Accession # KP453785) a newly- isolated laccase- synthesizing bacteria, which possesses a high similarity with *Bacillus songklensis*, has not yet been characterized. SS4 was isolated from a forest soil and has been reported to produce laccase efficiently without the catabolic repression by a higher concentration of glucose and yeast extract in the culture media (Kaur et al. 2016). In the present study, the compatibility of SS4 laccase with natural and synthetic mediators has been evaluated, the ammonium sulphate concentrated enzyme suited to industrially prefer harsh conditions has been characterized, and the enzyme production and application in the decolorization of indigo carmine dye, in the presence and absence of laccase mediators, has been optimized.

**Materials and Methods**

*Culturing conditions of the microorganism*

Laccase producing bacteria (*Bacillus* sp. SS4) was used in the present study. Bacterial culture was routinely prepared in M162 liquid or agar medium (Degryse et al. 1978) and the culture was maintained in a suspension of 30% glycerol at -70 °C. 1.0 % overnight grown SS4 was used to inoculate the M162 liquid broth and incubated at 37 °C at 150 rpm shaking condition for 48 h.

*Induction of laccase in SS4 by CuSO₄*
The stimulating effect of CuSO₄ on laccase production was observed by adding different concentrations (100 to 500 µM) of CuSO₄ separately to M162 liquid broth.

Ammonium sulphate precipitation of enzyme

The supernatant was obtained after centrifugation of M162 culture broth (1.0L) at 10,000 x g, 4 °C for 10 min and was used as a crude extracellular enzyme. The laccase was concentrated by ammonium sulphate precipitation of enzyme (40-80% saturation). The precipitated proteins were dialyzed for 8 h in 0.1 M phosphate buffer (pH 7.0) at 4 °C.

Enzyme assay

Laccase activity was determined by using 2 mM guaiacol (ε = 48,000M⁻¹ cm⁻¹, λmax = 465 nm) in 100 mM phosphate buffer (pH 7.0) at 75 °C for 15 min. Enzyme units were calculated in nkat (nmol of substrate converted/sec/ml of enzyme) (Singh et al. 2007). All the experiments were carried out in triplicate.

Effect of temperature on laccase activity and thermo stability

The effect of temperature on laccase activity was determined by performing the enzyme assay at different temperatures (20-90°C). The thermostability of the enzyme was evaluated by keeping the laccase at different temperatures (70, 80 and 90 °C) for 0 to 5 h at the optimum working pH of the enzyme and then the remaining activity of laccase was calculated.

Effect of pH on laccase activity

To ascertain the optimum pH of the enzyme, the laccase activity was determined by performing the enzyme assay at different pHs: 3.0 to 5.5 (0.1 M acetate buffer), 6.0 to 7.5 (0.1 M phosphate buffer), 7.5 to 9.0 (0.1 M tris buffer), and 8.5 to 10 (glycine, NaOH buffer).
**Effect of redox mediators on laccase activity**

The effect of different redox mediators (acetosyringone, vanillin, orcinol, veratraldehyde, syringaldehyde, hydroquinone, pyrogallol, \(p\)-coumaric acid, anthranilic acid, sinapic acid, ferulic and veratic acid, HBT and ABTS) on the laccase activity was determined by mixing each mediator (2 mM) separately with the enzyme at room temperature for 0-3 h at pH 7.0. The activity was measured by the standard procedure.

**Effect of high concentration of metals on laccase activity**

The effect of different metal salts NaCl (50-2000 mM), MgSO\(_4\), MnSO\(_4\) (10-40 mM) and CuSO\(_4\) (100-1600 µM) on laccase activity was determined by adding these salts in a standard reaction mixture of enzyme assay.

**Effect of high concentration of organic solvents on laccase activity**

The effect of different solvents (methanol, acetone, ethyl acetate and DMSO) 5.0-35 % (v/v) on the laccase activity was determined by carrying out the enzyme assay in the presence of the different solvents separately.

**Detection of laccase isoforms**

Electrophoresis (native-PAGE) was carried on 10% polyacrilamide gel (Laemml, 1970). Laccase was zymographed (visualization of laccase activity) by incubating the native gel in 2 mM guaiacol, prepared in 0.1 M phosphate buffer (pH 7.0) at 65 ºC for 20 min.

**Decolorization of indigo carmine dye**
Laccase (20 nkat) was applied for evaluating the potential of the enzyme to decolorize indigo carmine (50 µM) dissolved in phosphate buffer (pH-7.0) at 75 °C for 4 h, in the presence and absence of natural (acetosyringone and syringaldehyde) and synthetic (HBT and ABTS), redox mediators (100-500 µM).

*Statistical analysis*

All the experiments were carried out in triplicate and the mean ± SD has been reported.

**Results and discussion**

The bacteria are easy to handle and required much less incubation time for the laccase production (Sharma et al. 2007). Newly-isolated *Bacillus* sp. SS4 produces extracellular laccase that can quickly oxidize substrates such as syringaldazine, guaiacol and ABTS (Kaur et al. 2016). Syringaldazine is a non-auto-oxidative substrate which does not give a reaction with tyrosinase, lignin peroxidase or with hydrogen peroxidase and has been considered to be uniquely a laccase substrate (Harkin et al. 1974). The complete characterization was carried out with ammonium sulphate precipitated enzyme. Maximum laccase activity was found in the fraction precipitated with an 80 % saturation of ammonium sulphate.

*Induction of laccase by CuSO₄*

The addition of CuSO₄ (100 µM) in the production media stimulated the laccase synthesis by SS4 up to 8.5 fold without increasing the growth of bacterial cells. Laccase production by SS4 was constitutive but found very less devoid of copper, (Fig. 1). 500 µM CuSO₄ in the production media inhibited the growth and laccase synthesis in SS4. At a higher concentration, CuSO₄ acts as a potent inhibitor of bacterial growth and is used as a key
component in several antibacterial pesticides. Copper (Cu) has a dual role in the biological systems of living organisms. It acts as an essential trace element, working as a cofactor for several biocatalysts, including superoxide dismutase (providing protection against free radicals), cytochrome c oxidase (mitochondrial electron transport chain), and tyrosinase (pigmentation). At the same time, copper is toxic to microorganisms and may lead to the death of microorganisms within minutes of their exposure to the metal (Krumova et al., 2012). Copper is extremely toxic to bacteria, even slightly above the concentration normally present in those regions from where they are isolated (Wilson and Ray, 1956).

The addition of inducers to the production media generally increased the production of laccase at the level of gene transcription. The promoter regions of the genes encoding for laccase contain various recognition sites that are specific for xenobiotics and heavy metals. It has been noticed that Pleurotus ostreatus laccase genes poxc and poxa1b were transcriptionally induced by CuSO₄ and several putative metal responsive elements (MREs) were found on the promoter regions of these genes (Faraco et al., 2003; Sadhasivam et al. 2008). In the case of Lentinus crinitus, after the addition of CuSO₄ (150 µM) to the production media, the laccase synthesis increased (14,320 UL⁻¹), and the mycelial growth was reduced (Valle et al. 2014).

Effect of pH, temperature and thermostability of SS4 laccase

The optimum working pH of SS4 laccase was 7.0. The enzyme was ~90% stable at pH 7.0 up to 4.0 h and, at the same pH, laccase showed a 50% activity after 48 h. SS4 laccase showed the best working temperature within the range, 70-80 °C. The enzyme showed maximum stability at 70 °C with a 90% retaining activity after 5.0 h. Laccase activity of 88 and 23 % was retained at 80 and 90 °C respectively, after 3.0 h. The thermostability of enzymes is an attractive feature for their biotechnological applications (Berka et al. 1997; Singh et al. 2011). Laccases are
considered to be moderately thermostable and several attempts have been made to isolate highly thermostable laccases. Kiiskinen et al. (2004) isolated novel laccases from wood rotting fungi, with most having \( t^{1/2} \) of 3-6 h at 60 °C, but the pH optima of these laccases were 2.0-4.0. Reiss et al. (2011) reported laccase from *Bacillus pumilus* that lost 50% activity after 1h 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 a broad pH stability in both acidic and alkaline conditions (Zhang et al. 2013). The fungal laccases commonly possessed a lower thermostability than the bacterial laccases (Hildén et al. 2007).

**Effect of redox mediators on laccase activity**

A total of 14 laccase mediators were screened for evaluating their compatibility with laccase (Fig. 2A and 2B). It was found that acetrosyringone, vanillin, orcinol and veratraldehyde were entirely compatible with the enzyme and showed no adverse effect on the laccase activity after 3 h. Syringaldehyde, \( p \)-coumaric acid, ferulic acid and hydroquinone reduced the enzyme activity ≥50% after 1.0 h, but laccase activity remained at 100 to ~120 % in the presence of synthetic mediators, HBT and ABTS. Indeed, inactivation of laccase by the oxidized species of mediators is a major drawback of the laccase-mediator system (LMS). HBT has also been shown to inactivate (50% in 4 h) the laccase, and possesses a high toxicity even at low concentrations. HBT radicals inactivated laccase by the oxidation of aromatic amino acid residues on the protein surface (Ibarra et al., 2006). In the present study, natural mediators (syringaldehyde, ferulic acid and \( p \)-coumaric acid) reduced half of the laccase activity which is quite different from the general trend; natural mediators boosted the laccase activity (Halaburgi et al., 2011; Pardo et al. 2013; Nguyen et al. 2016). It has been proposed that natural mediators can be more efficient, cost effective and ecofriendly. Therefore, an extensive search is required to identify and screen
more economical and non-toxic natural mediators (Camarero et al. 2007). In the present study, the inhibitory effect of natural mediators on laccase activity was not understood. The mechanism for substrate oxidation by natural mediators has been studied for several years, however it has not been fully elucidated yet and this is still controversial (Torres-Duarte et al. 2011). Quantitative structure activity relationship (QSAR) studies have been frequently applied in biological and medicinal chemistry experiments, but studies on LMS are scarce. This is due to a series of limitations associated with QSAR studies in LMS, which are principally related to the heterogeneity of available experimental data (Dudek et al. 2006). In various studies, different reaction conditions were employed, such as substrate/mediator molar ratio, source of laccase, nature of substrate and reaction time. In addition, different activity rate measurements have been used; as well a limited number of mediators have been studied. This heterogeneity of the available data is the main limitation for comparison purpose (Medina et al. 2013). Although natural mediators represents the ecofriendly and less toxic alternatives to the synthetic (including -NOH-type) mediators for accelerating and broadening the spectrum of laccase based different applications, but major drawback of the natural mediators is grafting reaction during the delignification of pulps. At mill scale, biobleaching with natural mediators is not considered as cost-effective, until an extra step of washing is required with H₂O₂ or NaOH is needed for increasing the brightness of pulp (Singh et al., 2015)

Effect of metal salts on laccase activity

MgSO₄ and MnSO₄ (40mM) increased the enzyme activity up to 3.5 fold (Fig. 3A), laccase retained ~70 % activity at 2 M concentration of NaCl (Fig. 3B) and CuSO₄ (200µM) also enhanced the enzyme activity by 20 % (Fig. 3, C). In the case of γ-proteobacterium JB, Singh et al. (2009) reported that laccase activity was reduced ~50 % in the presence of 500 mM of NaCl. Zilly et al. (2011) reported that laccase from Ganoderma lucidum was inhibited >70 % in the
presence of 500 mM of NaCl. Zhang et al. (2013) noticed that NaCl is potent inhibitor of laccase from *Bacillus vallismortis*, with the activity of the enzyme being reduced up to 80% in the presence of 1000 mM of NaCl. Halaburgi et al. (2011) found that MnCl$_2$, MgCl$_2$ and CuSO$_4$ (1.0 mM) did not show any adverse effect on laccase from *Cladosporium cladosporioides*. Laccase from *Shiraia* sp.SUPER-H168 showed a small inhibitory effect of Cu$^{2+}$, Mn$^{2+}$ and Mg$^{2+}$ (10 mM) on enzyme activity: 5.0, 8.0 and 0.5% respectively (Yang et al. 2013). *Trematosphaeria mangrovei* showed a reduction in laccase activity of more than 10 and 30% in the presence of 1.0 mM MnSO$_4$ and MgSO$_4$, respectively (Atalla et al. 2013). Metal salts are present in very high concentrations in the effluents of textile and paper industries, and consequently there is great interest in oxidative enzymes that can bear the high level of salt concentrations during the bioremediation of effluents in the textile and paper industries (Zilly et al. 2011; Singh et al. 2015).

*Effect of organic solvents on laccase activity*

In the present study, 5.0-10% ethyl acetate enhanced the laccase activity by >10%. The enzyme retained 75, 75 and 50% activity in 10% methanol, acetone and DMSO, respectively. Activity of spore laccase from *Bacillus subtilis* WD23 was inhibited by 1.0 mM, methanol, ethyl acetate and acetone by 97, 18 and 15% respectively (Wang et al. 2011). Yang et al. (2013) reported that the activity of laccase from *Shiraia* sp.SUPER-H168 was reduced by 10 %, methanol and acetone to 8.0 and 17 % respectively. Pre-treatment of lignocellulosic biomass with organic solvents is recognized as the emerging process for fractionating the biomass to cellulose, lignin and hemicellulose components with a high purity and easy solvent recovery for reuse. Some studies showed that pre-treatment of biomass with organic solvents were needed to remove these before enzymatic treatments, especially in the case of the pulp and paper industries.
If such oxidative enzymes will be available that could bear the harsh effect of solvents, then there is no need of excessive washing of biomass with water (Zhang et al. 2016).

Decolorization of indigo carmine

The decolorization potential of SS4 laccase was evaluated by adding laccase to indigo carmine alone, and the mediator containing the reaction mixture. It was observed that SS4 laccase decolorized the dye in the absence of mediators at a very slow rate (~25% in 4.0 h). However, in the presence of mediators, the rate of decolorization was increased. Among the mediators used, acetylsyringone acted the most efficiently as it decolorized 100% of the dye in <10 min. 100% decolorization was also observed in the presence of synrigaldehyde and ABTS after 2.0 and 3.0 h, respectively. On the other hand, increasing the concentration of mediators also accelerated the rate of decolorization. On the contrary, enhancing the concentration of HBT decreased the rate of decolorization. 100 µM HBT was found to be the optimum, as it decolorized the indigo carmine completely in 3 h (Table 1). Singh et al. (2007) reported that mediators such as syringaldehyde, vanillin, p-hydroxybenzoic acid and HBT were used alone, and in combinations, to increase the decolorization of indigo carmine with laccase from γ-proteobacterium JB. Syringaldehyde > p-hydroxybenzoic acid > and vanillin increased the degradation and HBT had no effect. Cho et al. (2011) reported the degradation of indigo carmine by spore bonded laccase from Bacillus subtilis, with the complete degradation of the dye taking >3 h. Recently, Li et al. (2015) observed the decolorization (87%) of indigo carmine by adding it to the cultural broth of Bacillus sp. MZS10. They found that indigo carmine degradation took 15 h in a 5.0 L stirred-tank. Furthermore, they proposed a novel reductive mechanism of dye degradation in which the end product was indoline sulfonic acid. In the case of indigo carmine degradation by laccases via an oxidative pathway, the synthesis of isatin or isatin sulfonic acid were the end products (Fig. 4). Laccases from Trametes hirsuta and Sclerotium rolfsii were able to oxidize the indigo carmine to isatin (indole-2, 3-dione), which was further decomposed to anthranilic acid (Campos et al., 2001). The laccase from γ-proteobacterium JB degraded indigo carmine to anthranilic acid via isatin as determined by HPLC analysis (Singh et al., 2007). The Paenibacillus larvae laccase degraded indigo carmine to isatin sulfonic acid and anthranilic acid (Mohandass et al. 2008).
Detection of laccase isoforms

Copper sulphate (100 μM) induced laccase production in SS4 but did not trigger the synthesis of any isoform, only one band of laccase was observed on native PAGE irrespective of whether the enzyme was produced in the presence or absence of CuSO₄ (Fig. 5). In recent studies of bacterial laccases there has been no report of laccase isoforms, even in the presence of xenobiotics or metal inducers. A mixture of ferulic acid and vanillin induced the synthesis of laccase isoforms lcc1, lcc2 and lcc3, in the case of Pleurotus pulmonarius culture filtrates. The lcc1 and lcc2 isoforms were produced by non-induced cultures, while lcc3 was found only in the induced-culture filtrates (Desouza et al. 2004). The characterization of three laccase isoforms from Pleurotus nebrodensis were described as Lac1, Lac2 and Lac3 and purified to homogeneity by using different purification steps (Yuan et al. 2016).

Conclusions

Laccase from SS4 in combination with acetylsyringone decolorized the indigo carmine rapidly (<10 min) i.e. unprecedented in the literature. The laccase studied has the potential to replace the stone washing process (beating of jeans by pumice stones for removing indigo and producing contrasting effects) with an enzymatic mechanism, but this needs proper adequate industrial trials. The enzyme studied is thermo and halo tolerant and able to retain the activity in the presence of commonly existing industrial pollutants (NaCl, MnSO₄, MgSO₄ and CuSO₄). This result suggests the practical application of laccase in the bioremediation of effluent generated in the pulp and paper industries, which contain high levels of salt concentration. In the present work, the effect of different solvents on the enzyme activity may be of interest, since most aromatic pollutants can only be dissolved in organic solvents. Industries such as textile and paper are looking for highly stable laccases that can tolerate alkali, thermo and halo tolerant conditions.
with high specific activity, and which can work efficiently in the presence of a wide range of laccase mediator systems (LMS). Plausibly, SS4 laccase is highly compatible with a wide range of cost effective natural and synthetic mediators which indicates that this laccase is suitable for a wide range of industrial applications.

Acknowledgments: The authors are thankful to SERB/DST, Delhi, India, for providing the research funding under the Fast Track Young Scientist Program (SB/FT/LS-315/2012).

References


Liu Y and Hua X (2014) Degradation of acenaphthylene and anthracene by chemically modified laccase from Trametes versicolor. RSC 4: 31120–31122


Table 1: Decolorization of indigo carmine (50µM) by laccase in presence and absence of mediators

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<tr>
<td>Laccase</td>
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<td>6.0</td>
<td>6.0</td>
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<td>25</td>
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<tr>
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<td>20</td>
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<td>10</td>
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<td>86</td>
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Fig. 1: Induction of laccase synthesis in *Bacillus* SS4 by CuSO₄.

Fig. 2, A. Compatibility of laccase with mediators (natural and synthetic).
Fig. 2, B. Less, compatibility of laccase with mediators.

Fig. 3: Effect of metal salts on laccase activity (100% activity was, 35 nkat).

Fig. 4: Oxidative pathway of indigo carmine degradation (Li et al., 2015).

Fig. 5: Native, PAGE for the detection of laccase isoforms, lane A laccase was produced in the presence of (100 µM) CuSO₄; lane B laccase was produced in the absence of CuSO₄.
Fig. 1
Fig. 4

Indigo carmine → Oxidation → Isatin → Isatin sulfonic acid