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Full Paper

Performance of an alkalophilic and halotolerant laccase from γ-proteobacterium JB in the presence of industrial pollutants

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An alkalophilic and halotolerant laccase from γ-proteobacterium JB catalyzed in high concentrations of organic solvents and various salts. The enzyme retained 80–100% activity in 10% concentration of dimethylsulfoxide (DMSO), ethanol, acetone or methanol; 100, 85 and 50% activity in 20 mM $MgCl_2$, 5.0 mM $MnCl_2$ and 0.1 mM $CuCl_2$; 140, 120 and 110% activity in 5.0 mM $MnSO_4$, 10 mM $MgSO_4$ and 1mM $CaSO_4$, respectively. Sodium halides inhibited the enzyme in the order: $F^- > Br^- > I^- > Cl^-$. In 0.5 M NaCl, pH 6.0, laccase was ~60% active. Decolorization of indigo carmine by laccase at pH 9.0 was not inhibited even in the presence of 0.5 M NaCl. Release of chromophoric, reducing and hydrophobic compounds during biobleaching of straw rich-soda pulp by laccase was not inhibited when the enzyme was applied in the presence of 1 M NaCl at pH 8.0. Laccase retained 50% residual activity even when incubated with 5% calcium hypochlorite for 30 min.

Key Words——alkalophilic; bleaching; chlorine; halotolerant; indigo; laccase; γ-proteobacterium; solvents

Introduction

Laccases (benzenediol oxygen oxidoreductases, EC 1.10.3.2) are polyphenol oxidases that require O_2 to oxidize phenols, polyphenols, aromatic amines and different non-phenolic substrates by one electron transfer resulting in the formation of reactive radicals (Gianfreda et al., 1999). They are members of the multicopper protein family that has developed from small sized prokaryotic azurins to eukaryotic ceruloplasmin. Laccases are widely distributed in plants and fungi where their involvement in melanin formation and in a variety of different, and sometimes contradictory, physiological functions like fungal morphogenesis, plant pathogenesis and fungal virulence has been frequently proposed. They also occur in prokaryotes e.g. *Azospirillum lipoferum*, *Marinomonas mediterranea*, *Bacillus subtilis* spore and γ-proteobacterium JB (Bains et al., 2003). In natural ecosystems these enzymes oxidize phenolic compounds from lignin while reducing oxygen to water. Laccases raised great interest because of the non-specific reactions they catalyze. They have been extensively used for transformation of aromatic compounds such as dyes, aromatic pollutants or in wastewater treatments (Singh et al., 2007). The laccase mediator system (LMS) has already found practical applications such as the Lignozym®-process for biobleaching of pulp. However, all these biobleaching studies were focused on wood pulps and little is known about the efficiency of the LMS on non-wood pulps (Singh et al., 2008). Laccases have been proposed to activate the fiberbound lignin during the manufacturing of composites, thus, resulting in boards with good mechanical properties without toxic synthetic adhesives. Since laccases are able to catalyze elec-

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tron transfer reactions without additional cofactors, their use has also been studied in biosensors to detect various phenolic compounds, oxygen or azide. Moreover, biosensors for the detection of morphine and codeine, catecholamines and also for electroimmunoassay have been developed. Thus, laccases are able to mediate coupling, reducing 2,4,6-trinitrotoluene (TNT) metabolites to an organic soil matrix, which results in detoxification of munition residue. Moreover, PAHs, which arise from natural oil deposits and utilization of fossil fuels, were also found to be degraded by laccases (Couto and Herrera, 2006). Recently, cosmetic and dermatological preparations containing proteins for skin lightening have also been developed using laccases (Golz et al., 2004).

Very few bacterial laccases have been reported and characterized to date. Previously we isolated, purified and characterized a bacterial (γ-proteobacterium JB) alkalophilic laccase (Singh et al., 2007). This laccase was used for biobleaching of agrowaste pulp and as a result of enzyme bleaching approximately 10% reduction in the chlorine consumption was achieved (Singh et al., 2008). The main objectives of the present study were to analyze the performance of the alkalophilic and halotolerant laccase in the presence of industrial pollutants/chemicals. These chemicals are commonly used in various industries and are present in their effluents, e.g. textile industry, distilleries, paper and pulp industries. Decolorization of the textile dye indigo carmine and release of chromophoric, hydrophobic and reducing compounds from wheat straw rich-soda pulp by laccase was studied in the presence of a high concentration of NaCl. From our point of view it is essential to investigate whether such solvents and salts inhibit laccase activity and have an effect on its use in dye decolorization and pulp bleaching. Thus, this work explains more precisely the inhibition observed since a weaker activity may have various causes depending on experimental conditions.

Materials and Methods

Microorganism and cultural conditions. γ-Proteobacterium JB, isolated from industrial waste water and identified previously in our laboratory (Bains et al., 2003) was maintained as a suspension in 20% glycerol at −70°C and was routinely cultured on M162 medium (Degryse et al., 1978). One milliliter of inoculum (overnight culture) was used to inoculate 100 ml of M162 medium, incubated at 37°C and 150 rpm for 24 h. Culture supernatant obtained by centrifugation at 10,000 $\times g$, 4°C for 10 min was used as crude extracellular preparation with enzyme activity 7.8 nkat ml^{-1} and specific activity 65 nkat mg^{-1} protein.

Purification of laccase. Laccase was purified to homogeneity by ammonium sulphate precipitation, DEAE-Sepharose cation exchange chromatography and preparative Native-PAGE as described previously (Singh et al., 2007).

Enzyme assays. Bacterial laccase activity was determined using 100 μM syringaldazine as substrate, at 55°C in 0.1 M phosphate buffer (pH 6.5). The change in absorbance due to oxidation of syringaldazine in the reaction mixture was monitored at 525 nm (ε = 50,000 M^{-1} cm^{-1}) for 10 min of incubation. Enzyme units were expressed in nkat (nmol of substrate converted s^{-1} ml^{-1} of enzyme). Cellulase, xylanase, lignin peroxidase and manganese peroxidase were tested by the method of Miller et al. (1959) and Tien and Kirk (1988). All experiments were carried out at least in triplicate and the mean values are reported.

Decolorization of indigo carmine. For the decolorization experiment, indigo carmine concentration (100 μM) and laccase units (40 nkat) were kept constant and concentration of NaCl was varied from 0–4 M. Decolorization was performed for 4 h at 55°C and pH 9.0 (0.1 M,Tris-HCl buffer) (Singh et al., 2007).

Release of chromophores, hydrophobic substances and reducing compounds from wheat straw-rich soda pulp. First stage raw pulp (composed of w/w 78.8% wheat straw (*Triticum astivum*), 10.6% sarkanda (*Saccharum spontaneum*) and 10.6% candy (*Eragrostis* sp.) cooked at 165–175°C for 30 min at a pressure of 7.0–7.5 kg m^{-3}) was treated with laccase (20 nkat/g of pulp) and mediator (2 mM ABTS) in a baffled, 250 ml Erlenmeyer flask shaken at 250 rpm, 55°C, pH 8 for 4 h. Denatured and active laccase-treated pulp was filtered through muslin cloth and the filtrate was studied for release of chromophores, hydrophobic compounds and reducing compounds (Singh et al., 2008).

Stability of laccase in the presence of calcium hypochlorite. Thirty nkat of laccase was treated with various concentrations (1–10%) of calcium hypochlorite and after 10, 30 and 60 min the activity of the enzyme was checked by the standard assay method using syringaldazine as the substrate.

Results and Discussion

The organism used was Gram-negative, non-sporulating, non-hemolytic, singly occurring, short rods. It did not grow on McConkey or cetrimide agar and was oxidase positive but catalase, indole, methyl red, Voges-Proskauer, and citrate negative. Growth on glucose was positive with no gas and no acid production. 16S rDNA sequencing of γ-proteobacterium JB was carried out previously in our laboratory. A BLASTn algorithm search of GenBank database (http://www.ncbi.nlm.nih/BLAST) exhibited 98% identity with the closest match, γ-proteobacterium F8. So, the present organism was named γ-proteobacterium JB (Accession No. AF 542077). Recent analysis showed that γ-proteobacterium JB belongs to the genus *Rheinheimera*. The bacterium produced laccase but was not found to produce cellulase, xylanase, lignin peroxidase or manganese peroxidase enzymes even when tested in 100 times concentrated cell-free supernatant and intracellular preparations.

Effect of solvents

The enzyme showed 100, 90, 90 and 80% activity in 10% concentrations of DMSO, ethanol, acetone and methanol, respectively. Ethylacetate and isopropanol reduced the activity by 90 and 95%, respectively. Laccase showed approximately 50% activity even in 40% concentration of ethanol (Fig. 1). The effect of high concentrations of solvents, various salts and chemicals on laccase was studied because this characterization will open new possibilities to apply this enzyme in various industries like pulp and paper, denim bleaching, beverage and treatment of textile effluent contaminated with dyes. Various enzymes, irrespective of their functional activities (hydrolytic/oxidative), show resistance to polar solvents. This resistance depends upon the polarity index and denaturation capacity of the solvent (Gupta et al., 1997). Figure 1 shows that activity of laccase, in general, decreased with decreasing polarity index of solvents. Polarity index is the best guide among all solvent parameters for selecting solvents in which the enzymes are relatively more stable. This parameter may help find the best solvent for different applications of this laccase.

Laccase with improved solvent tolerance would be beneficial for potential oxidation of organosulfur compounds in petroleum to reduce viscosity and improve the quality of petroleum (Van et al., 2003). Olive mill wastewater is a phenolic-rich effluent and hence a good candidate for degradation by laccases. Borja et al. (1992) proposed that use of fungal species in two-stage fermentation may help the biodegradation of olive mill wastewater. Laccase may also prevent the undesirable changes such as discoloration, clouding, haze or flavor changes in beer, fruit juices and wine, improving their shelf life by removing phenols such as coumaric acids, flavans and anthocyanins (Couto and Herrera, 2006). The effect of solvents on laccase activity may be of great interest since most aromatic pollutants can only be dissolved in organic solvents (Farnet et al., 2008). Thus involvement of laccase in xenobiotic transformation has to be monitored using co-solvents and sometime using biphasic reaction mixtures (Bog-

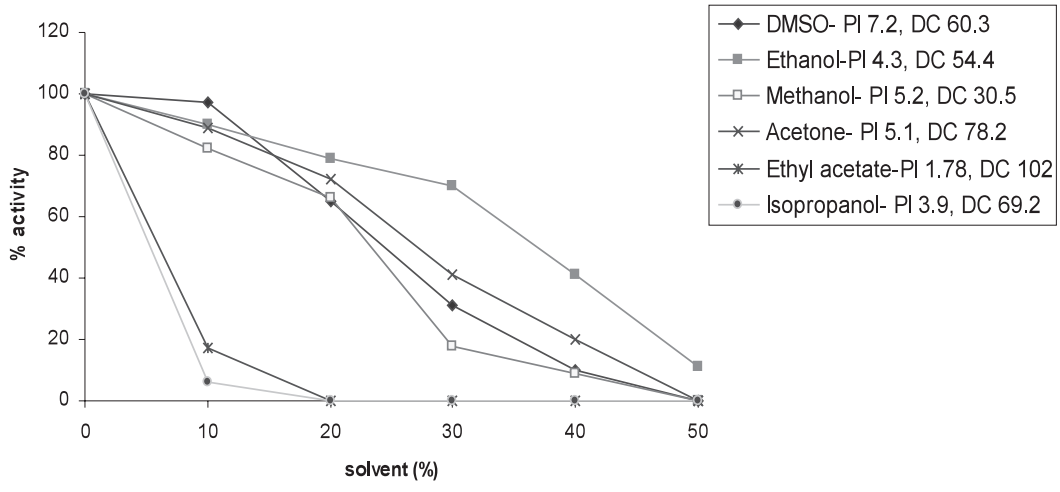

Fig. 1. Effect of solvents on laccase activity (100% activity=50 nkat). PI is polarity index, DC is denaturation capacity (Gupta et al., 1997).

danovskaya et al., 2002).

Effect of metal chlorides

Effect of chloride salts on laccase activity showed that enzyme was 100, 85 and 50% active in the presence of 20 mM $MgCl_2$, 5 mM $MnCl_2$ and 0.1 mM $CuCl_2$ (or $CaCl_2$), respectively. 0.1 mM of $HgCl_2$ inhibited the laccase activity completely. At 5.0 mM, $MgCl_2$ enhanced the laccase activity by 10–12% (Fig. 2). Effluents from textile dyeing industries contain metals used in dye production technologies or in the molecule of the dyes. The interaction of metals with extracellular ligninolytic enzymes of white-rot fungi and laccases is particularly important for the understanding of the regulation of biotechnological processes of fungal degradation of xenobiotics (Couto et al., 2005). The laccase from *Pleurotus ostreatus* and *Hyphomycete chalara* was highly sensitive to Hg^{2+}; indicating the presence of thiol groups essential for laccase activity (Baldrian and Gabriel, 2002; Robles et al., 2002).

Effect of metal sulphates

$CuSO_4$ inhibited the laccase activity by 60% at 0.1 mM, but laccase activity was enhanced by 40, 20 and 10% in the presence of 5 mM $MnSO_4$, 10 mM $MgSO_4$ and 1 mM $CaSO_4$, respectively. The enzyme was more than 90% active in 50 mM of $MgSO_4$ (Fig. 3). Enhancement of laccase production on $CuSO_4$ addition to fungal or bacterial culture has been extensively described (Malhotra et al., 2004; Lorenzo et al., 2006) which may be explained by gene-expression induction. However, when added to purified enzyme, $CuSO_4$ induced varied effects depending on the laccase tested. Lorenzo et al. (2005) showed 40% inhibition of *Trametes versicolor* laccase with 20 mM of $CuSO_4$ while Baldrian and Gabriel (2002) showed that laccase activity increased and remained stable with a $CuSO_4$ concentration of 50 mM.

Effect of Na-halides

Concentrations of Na-halides, where 50% of laccase activity remained (IC_{50}), were 10 mM for NaF, 30 mM for NaBr and 50 mM for NaI, while laccase was more than 60% active at 500 mM concentration of NaCl (Fig. 4). Due to high stability in NaCl, laccase will find preference for use in various industrial processes where high concentration of chlorine is needed frequently, e.g. in biobleaching of pulp. Mechanisim of inhibition of laccase by halides has been described by Naki and Varfolomeev (1981). Cl^- and Br^- ions acted as competitive inhibitors with the electron donor while F^- acted as a non-competitive inhibitor. Furthermore, the degree of inhibition of laccases by halides seems to be linked to the availability of copper atoms (type 2 and type 3) in the active site. Laccase from γ-proteobacterium JB posessed only type 1 copper atoms (Singh et al., 2007) and retained ~60% activity even at 0.5 M NaCl, revealing the probability that Cu^{2+} centers may not be inhibited/available to excess Cl^- ions.

Decolorization of indigo carmine

0.2 M NaCl increased indigo carmine degradation by 25%. The presence of 0.5 M NaCl resulted in the same degradation as without NaCl (Fig. 5). Degrada-

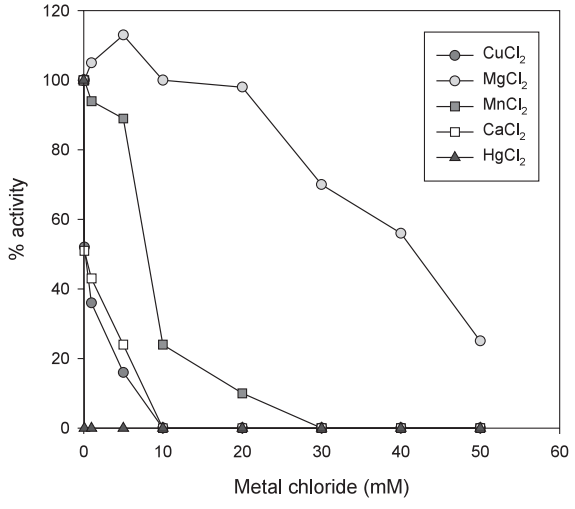

Fig. 2. Effect of metal chlorides on laccase activity (100% activity=39 nkat).

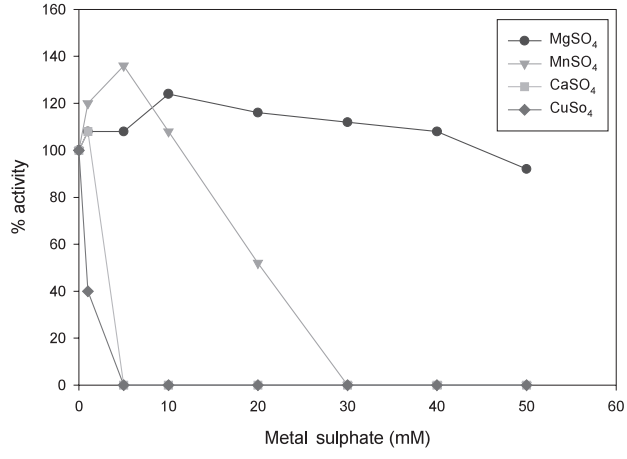

Fig. 3. Effect of metal sulphates on laccase activity (100% activity= 25 nkat).

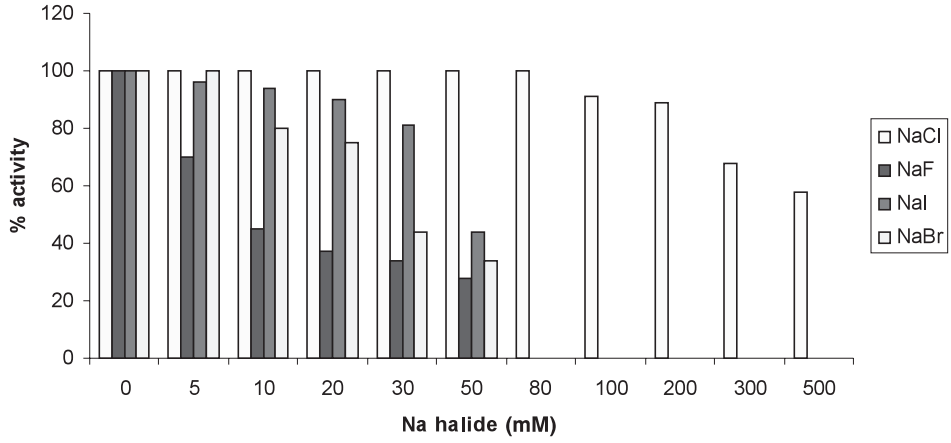

Fig. 4. Effect of sodium halides on laccase activity (100% activity=60 nkat).

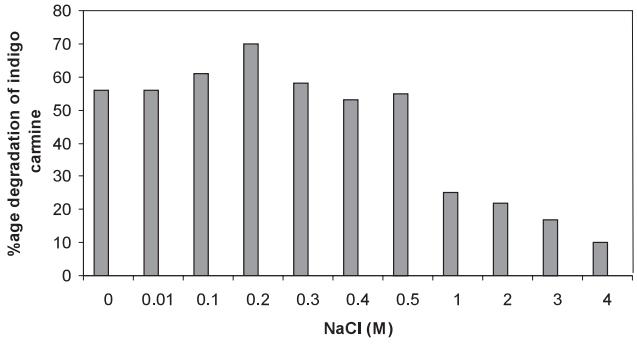

Fig. 5. Effect of NaCl on degradation of indigo carmine by laccase (40 nkat) from γ-proteobacterium JB.
100% color=1 O. D. at 610 nm.

tion of indigo carmine by laccase in the presence of a high concentration of NaCl may prove beneficial to the denim textile industries and for detoxification of effluent waste, where NaCl is present in excess with other metal salts. Laccase from γ-proteobacterium JB degraded indigo carmine to anthranilic acid by the same pathway as followed by fungal laccase (Singh et al., 2007).

Release of chromophores, hydrophobic and reducing compounds from soda pulp

Treatment of raw pulp with enzymes like xylanases or laccases releases these compounds, indicating the removal of non-cellulosic materials like hemicellulose (reducing compounds) and lignin (chromophores and hydrophobic compounds). γ-Proteobacterium JB laccase released these compounds and release was not affected even in the presence of 1.0 M NaCl (Table 1). This is the first report on an alkalophilic and halotolerant bacterial laccase used for biobleaching of agro-

Table 1. Effect of 1 M NaCl on laccase activity on wheat straw rich soda pulp at pH 8.0, temp. 55°C for 4 h.

Pulp treatment	Reducing sugars $OD_{550\ nm}$	Hydrophobic compounds $OD_{465\ nm}$	Chromophores $OD_{237\ nm}$
Raw pulp+ 1 M NaCl	0.65	0.60	3.1
Raw pulp+ ABTS+ boiled X + 1 M NaCl	0.66	0.62	3.0
Raw pulp + ABTS + X	2.1	1.4	6.2
Raw pulp + ABTS + X+ 1 M NaCl	2.0	1.5	6.1

X: Laccase (20 nkat g^{-1} pulp).

waste wheat straw-rich soda pulp, indicating that laccase can perform in the presence of a high concentration of chlorine.

Stability of laccase in calcium hypochlorite

Calcium hypochlorite is widely used in the pulp and paper industries for bleaching of pulp due to its low cost and easy availability. Laccase was ~90 to100% stable in 1–2% of calcium hypochlorite for 10 min. At 5% hypochlorite, enzyme was more than ~50% stable even after 30 min (Fig. 6), indicating that this enzyme has the potential to work in the presence of the 5% hypochlorite concentration usually employed in pulp bleaching industry. Singh et al. (2008) reported treatment of pulp (after hypochlorite (H1H2) stage) with xylanase and laccase only after the extensive washing

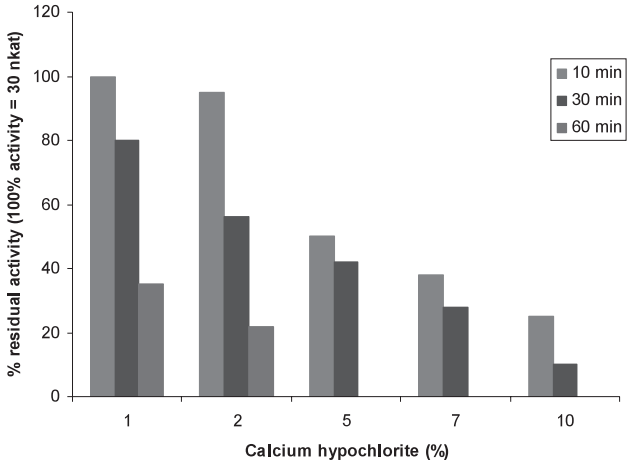

Fig. 6. Stability of laccase in the presence of calcium hypochlorite.

Residual activity was measured by standard assay as described in MATERIALS AND METHODS.

with water so as to remove hypochlorite. Stability of laccase in calcium hypochlorite eliminates the need for washing, thus reducing the need for water. It is interesting to note that Ca^{2+} ions generated from $CaCl_2$/$CaSO_4$ in water inhibited laccase even at low concentration, whereas in the presence of $Ca(OH)_2$ (feebly ionized), generated from calcium hypochlorite in water, laccase did not lose much of the activity.

Conclusion

A new bacterial laccase has been characterized by evaluating its activity/stability in the presence of common industrial pollutants (chlorides, sulphates, halides, solvents and calcium hypochlorite). Results indicate that this laccase has the potential to be used for denim stone washing and bleaching where various metal salts are used in excess. This laccase may also be useful in bio-pulping, and biobleaching of pulp, where chlorides and hypochlorites are applied extensively for lignin removal. The effect of various solvents on laccase activity may be of interest since most aromatic pollutants can only be dissolved in organic solvents

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