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Extracellular Laccase from *Pleurotus ostreatus* Strain EM-1: Thermal Stability and Response to Metal Ions

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ABSTRACT

The metal ion response profile and thermal stability of extracellular laccase from *Pleurotus ostreatus* strain EM-1, which is widely cultivated in Ghana, were investigated to provide information essential for the establishment of laccase-based applications in the country. *P. ostreatus* (Jacq. ex. fr) Kummer strain EM-1 was cultivated on a mixture of *Triplochiton scleroxylon* (wawa) sawdust, rice bran and lime. Extracellular laccase was isolated from spent sawdust four to six days after the appearance of mushroom pinheads and subjected to ammonium sulphate precipitation and gel filtration using Sephadex G-75. Laccase activity was assayed spectrophotometrically at 468 nm using 2,6-dimethoxyphenol in Mcilvaine's citrate-phosphate buffer, pH 5.0. Two metal ions, Cu^{2+} and Mn^{2+}, stimulated *P. ostreatus* strain EM-1 laccase activity. Cu^{2+} caused a maximal stimulatory effect of 324.4%, while Mn^{2+} exerted a more moderate stimulatory effect of 180.5%. Magnesium ions had no effect on the activity of the enzyme. Activity decreased by 77.4% after 20 min of incubation at 50°C. During seven days of storage at either 4 or -20°C, laccase activity decreased by 87.7-88.8%. This rate of deactivation was reduced to 28.1-32.8% over the same period when 20 mM $CuSO_4$ was added to the enzyme prior to storage. The findings suggest that *P. ostreatus* strain EM-1 laccase would not be a suitable biocatalyst for high temperature processes. Furthermore, copper and to a lesser extent, manganese can be used as stimulatory additives during *P. ostreatus* strain EM-1 laccase-catalyzed processes at ambient temperature and for short-term storage of the enzyme.

Key words: *P. ostreatus* strain EM-1, laccase, metal ions, thermostability, storage

INTRODUCTION

Laccases (benzenediol:oxygen oxidoreductases [EC. 1.10.3.2]) are receiving considerable attention because of their potential industrial and biotechnological applications (Kunamneni *et al*., 2008; Mohidem and Mat, 2009; Grassi *et al*., 2011; Desai and Nityanand, 2011). They are members of the family of blue multicopper oxidases and are produced mainly by fungi but are also present in bacteria, plants and insects (Ghosh *et al*., 2008; Sarnthima and Khammuang, 2008; Bryjak and Rekuc, 2010; Das *et al*., 2011). These enzymes catalyze the oxidation of phenolic and other aromatic compounds with a concomitant reduction of molecular oxygen to water (Khammuang and Sarnthima, 2007; Munusamy *et al*., 2008a; Forootanfar *et al*., 2011; Garg and Tripathi, 2011). Typically, laccases are involved in a two-step oxidation of phenolic substrates. Although, the

mechanism of action of laccases is not fully understood, it is well-documented that initially, an aryloxy radical is produced in a one electron oxidation and, subsequently, the active intermediate is converted to a quinone.

The diverse biotechnological applications of laccases include detoxification of municipal waste and effluents from the petrochemical, food and textile industries; elimination of recalcitrant soil pollutants (Prabu and Udayasoorian, 2005; Cordi *et al.*, 2007; Ghidouche *et al.*, 2007; Wu *et al.*, 2008; Garcia *et al.*, 2011; Chiaiese *et al.*, 2011); bio-bleaching processes in the paper and pulp industry (Monje *et al.*, 2010; Grassi *et al.*, 2011; Moldes and Vidal, 2011) and bio-upgrading of crop residues (Mensah *et al.*, 2012). Clearly, the benefits to be derived by developing countries from the applications of laccases are immense. For instance, laccase technologies in the areas of detoxification and bio-upgrading would be advantageous to Ghana's emerging downstream oil sector as well as agro-waste management. The biggest impediment to the establishment of laccase-based technologies in developing countries is the availability of the enzyme (El-Shora *et al.*, 2008). In view of the fact that commercial cultivation of *P. ostreatus* strain EM-1 is well established in Ghana, a simple and scalable procedure for isolating extracellular laccase from spent substrate has recently been described to ensure the availability and affordability of the enzyme in the country (Adamafio *et al.*, 2008).

Wide differences exist in the biochemical and physicochemical properties of laccases from diverse sources. These include thermal stability and sensitivity to metal ion additives, which dictate the types of biotechnology applications to which they are best suited (Mansur *et al.*, 2003; Papinutti *et al.*, 2008; Munusamy *et al.*, 2008b; Lettera *et al.*, 2010). There is a paucity of information on these characteristics of *P. ostreatus* strain EM-1 laccase. The purpose of the present study was, therefore, to investigate the thermal stability and metal ion requirements of extracellular laccase isolated from *P. ostreatus* strain EM-1 during solid-state cultivation.

MATERIALS AND METHODS

Compost bags of *P. ostreatus* (Jacq. ex. fr) Kummer strain EM-1 ready for cropping were obtained from the CSIR - Food Research Institute, Ghana. 2,6–dimethoxyphenol (DMP) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Fluka BioChemika, Germany. (±) -Catechin hydrate was obtained from Sigma-Aldrich Chemie, Germany.

Mushroom cultivation and enzyme preparation: *P. ostreatus* (Jacq. ex. fr) Kummer strain EM-1 was cultivated on *T. scleroxylon* sawdust compost prepared by the outdoor single-phase solid waste fermentation. Rice bran and lime were added in proportions as described by Obodai *et al.* (2010). Extracellular laccase was isolated from spent sawdust four to six days after the appearance of pinheads as previously described (Adamafio *et al.*, 2008). Partial purification was achieved by subjecting the crude enzyme extract to ammonium sulphate precipitation, gel filtration chromatography on Sephadex G-75 as well as anion exchange chromatography on DEAE-cellulose. Apparent homogeneity on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis performed as described by Laemmli (1970) was taken as evidence of purity. In subsequent experiments, ion exchange chromatography was omitted as it did not contribute significantly to the purification factor. Determination of laccase activity over a pH range of 3 to 8 and temperature range from 30 to 70°C showed that the omission did not alter the previously observed pH and temperature optima.

Determination of laccase activity: Laccase activity was assayed spectrophotometrically at 468 nm using 2,6-dimethoxyphenol as substrate (Leite *et al*., 2003). One unit of laccase activity was defined as the amount of enzyme oxidizing one µmol of 2, 6-dimethoxyphenol per minute at pH 5.0 and 45°C. Protein concentration was determined by the Folin-Lowry method (Lowry *et al*., 1951).

Thermal stability of laccase: Thermal stability was assessed by incubating 0.5 mL aliquots of purified enzyme at the optimum temperature of 50°C in 60 mM Mcilvaine's citrate-phosphate buffer, pH 5.0, for varying time periods (up to 120 min) and subsequently measuring laccase activity for 5 min.

Effects of metal ions and glycerol on laccase activity: The sensitivity of *P. ostreatus* strain EM-1 laccase to metal ions ($CuSO_4$, $MnSO_4$, $MgSO_4$) at concentrations ranging from 0 to 25 mM was investigated. The rate of deactivation of the enzyme during storage for 7 days at either -20°C or 4°C was also determined. The influence of $CuSO_4$ (20 mM) on the rate of deactivation of laccase was also studied. The optimum concentration of glycerol necessary for storage of the enzyme was also investigated. The results are presented as means of eight determinations.

Statistical analysis: Analysis of Variance (ANOVA) tests along with Least Significant Difference (LSD) post-hoc comparisons were conducted using Excel Data Analysis Statistical Software and Statgraphics-plus Software Programme (Version 3.0). The level of significance was set to $p<0.05$. Differences between means with $p<0.05$ were accepted as being statistically significant.

RESULTS

P. ostreatus strain Em-1 laccase was successfully isolated from spent substrate and partially purified. The degree of purification achieved was 12.7-fold with a yield of 21%. As shown in Fig. 1, two of the three metal ions tested, copper and manganese, stimulated laccase activity significantly ($p<0.01$). Of the two, copper was more potent, exhibiting a maximal stimulatory effect of 324.4% compared with 180.5% by manganese. Magnesium was completely without effect. Maximal effects were produced by 20 mM $CuSO_4$ and 10 mM $MnSO_4$ (Fig. 1).

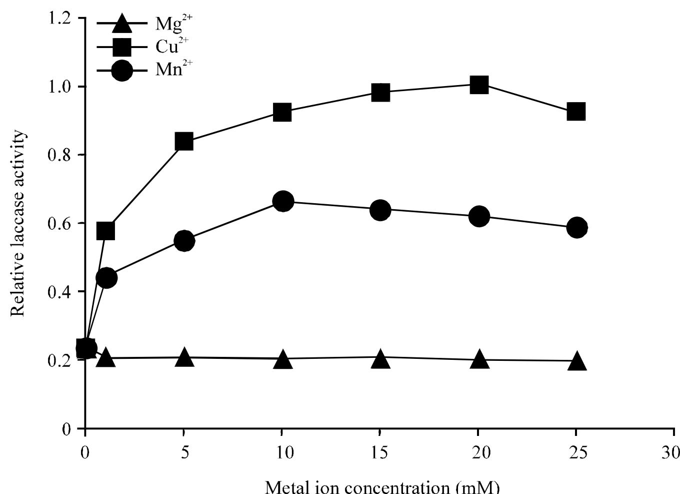

Fig. 1: Effects of metal ions on *P. ostreatus* strain EM-1 laccase activity Each point represents the mean of eight determinations

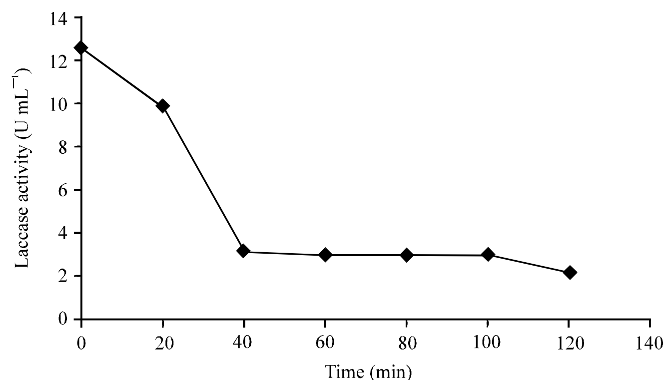

Fig. 2: Thermal stability of *P. ostreatus* EM-1 laccase at 50°C

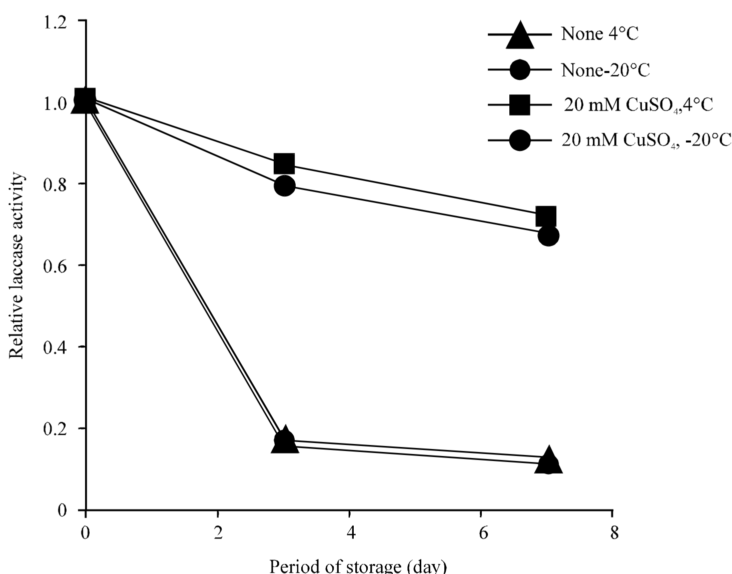

Fig. 3: Stability of *P. ostreatus* strain EM-1 laccase during cold storage

Studies on thermal stability showed that laccase activity dropped sharply by 77.4% after 20 min of incubation at 50°C (Fig. 2).

In the absence of exogenous metal ions, laccase activity decreased sharply by 87.7-88.8% over the 7 day storage period at 4 and -20°C, respectively (Fig. 3). Laccase activity declined gradually with time when samples were stored at either 4 or -20°C. The differences between laccase activity at the two temperatures were not statistically significant. This was to a large extent minimized (28.1-32.8% reduction in activity) by the addition of $CuSO_4$. The presence of glycerol (18%) during storage was found to be beneficial (Fig. 4).

DISCUSSION

Metal ion additives often enhance the performance of laccases in technological processes. Although many laccases respond positively to copper, magnesium and manganese, significant differences exist in responsiveness depending on the source of the enzyme. The importance of studying the characteristics of laccases from different sources is underscored by their striking

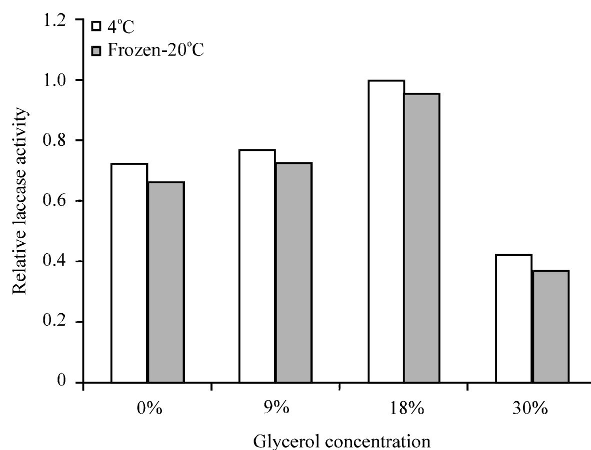

Fig. 4: Effect of glycerol on *P. ostreatus* strain EM-1 laccase activity

dissimilarities. It was, therefore, of prime importance to establish the response profile of *P. ostreatus* strain EM-1 laccase to these potential metal ion additives. The observed pronounced effect exerted by copper ions on laccase activity was in consonance with numerous reports on the metal ion requirements of laccases (Stajic *et al*., 2006; Papinutti *et al*., 2008; Phetsom *et al*., 2009). The generally positive response to copper is not surprisingly because laccases contain at least two types of copper centres; a mononuclear centre that serves as the site for substrate oxidation and a trinuclear centre where the reduction of oxygen to water occurs (Piscitelli *et al*., 2010; Bento *et al*., 2010; Desai and Nityanand, 2011). Despite this fact, not all laccases are stimulated by copper ions. For instance, laccase from *P. ostreatus* strain 10969 was reported to be inhibited by copper ions (Liu *et al*., 2009). The observed ability of manganese ions to activate *P. ostreatus* strain EM-1 laccase is in general agreement with the findings on laccases from other sources (Stajic *et al*., 2006; Papinutti *et al*., 2008). According to Lundell and Hatakka (1994), manganese ions, participate either as reducing (Mn(II)) or oxidizing (Mn(III)) agents in laccase-catalyzed reactions. The stimulatory effect on manganese on *P. ostreatus* strain EM-1 laccase contrasts sharply with the reported inhibition of *Daedalea quercina* laccase by Mn^{2+} (Baldrian, 2004). Interestingly, the activity of *Streptomyces cyaneus* CECT 3335 laccase was found to be enhanced by Mg^{2+} (Arias *et al*., 2003), which had absolutely no effect on *P. ostreatus* strain EM-1 laccase in this study.

A critical determinant of process parameters for laccase applications is the thermal stability of enzyme in question. The present studies on the thermal stability of *P. ostreatus* strain EM-1 laccase confirmed the previously reported optimum temperature value of 50°C (Adamafio *et al*., 2008) and revealed that the purified enzyme was highly thermolabile, retaining only 22.6% of its activity after 60 min of incubation at 50°C. This is in sharp contrast to the reported thermal stability of laccases isolated from a number of organisms including that from *S. cyaneus* which is reported to have retained more than 75% of its activity after incubation for 120 min at 50°C (Arias *et al*., 2003). Perhaps, the thermal instability of the purified *P. ostreatus* strain EM-1 laccase might be attributable to a low degree of glycosylation. As is the case with other glycoproteins, the thermal stabilization of laccases is directly proportional to the number of attached oligosaccharides, which typically consist of galactose, mannose and N-acetylglucosamine (Claus, 2004; Kunamneni *et al*.,

2008). It has been reported that the degree of protection from high temperature denaturation also depends on the position of the glycosylation sites (Shental-Bechor and Levy, 2008).

Experiments were also conducted to investigate the stability of the enzyme during short-term cold storage. It emerged that approximately 88% of laccase activity was lost after 7 days of storage at either 4 or -20°C. The rate of deactivation of the laccase was reduced to approximately 30% over the same period when $CuSO_4$ was added, at optimum concentration, to the enzyme prior to storage. It is likely that alterations in non-covalent interactions that led to changes in the conformation of the polypeptide chain and contributed to the deactivation that occurred during cold storage. Although catalytic activity at 4°C appeared to be slightly higher than it was at -20°C, these differences were not statistically significant. These findings clearly suggest that saturation of the enzyme with $CuSO_4$ prior to storage would be advantageous as it would drastically reduce the rate of deactivation. Polyols are known to stabilize protein structure and help preserve catalytic activity (Sousa, 1995; Papinutti *et al*., 2008). For this reason, the effect of glycerol on *P. ostreatus* strain EM-1 laccase during cold storage was also investigated. The present findings suggest that storage in the presence of 18% glycerol might be advantageous.

In conclusion, this study provides information critical to the correct choice of process parameters for the envisaged biotechnological applications of *P. ostreatus* strain Em-1 laccase in Ghana. Although the thermolabile nature of *P. ostreatus* strain EM-1 laccase raises questions about its suitability as a biocatalyst for high temperature processes such as biobleaching, it has been used successfully in the biodetoxification of cocoa pod husk (Mensah *et al*., 2012) and would probably be suitable for many ambient temperature applications.

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