Cloning and Expression of a new bacterial laccase from *Bacillus ligniniphilus* L1 DSM 26145T in *Escherichia coli* to understand enzymatic lignin depolymerization

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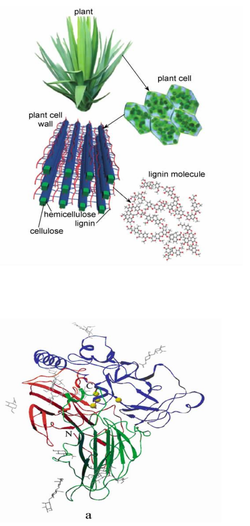
Introduction

Laccases (EC 1.10.3.2) as a member of multicopper oxidases have been found widespread in bacteria, fungi plant and insects. As laccases are nonspecific enzymes, its availability of substrates is broad. In addition, laccases with mediators such as ABTS (2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) together form a laccase-mediator system (LMS), which significantly enhanced the enzyme action and greatly enlarged the range of substrates. Therefore, the laccase have wider potential applications in biotechnology, including lignin degradation and modification, delignification of paper pulp, degradation of dyes and modification of synthetic and polymeric materials. In this study, we performed a heterologous expression and purification of a laccase gene derived from *Bacillus* L1 and elucidated its enzymatic properties. We used wood mill lignin and alkaline lignin to study the depolymerization characteristics of lignin by the bacterial laccase in the presence or absence of mediators using analysis techniques such as ToF-SIMS, GC-MS and GPC.



Objectives

(1) To elucidate the degradation of lignin by Lacc and its products.
(2) To initially establish a system for Lacc to degrade lignin.

Materials and methods

- Cloning, expression and purification of the Lacc gene
- Enzymatic characterization of *B.ligniniphilus* L1 DSM 26145T laccase
- Lignin discoloration
- GC-MS analysis
- ToF-SIMS analysis.

Results and discussion

Cloning, expression and purification of the Lacc gene

The Lacc protein showed a distinct band at approximately 60 kDa on SDS-PAGE (Figure 1a) and similar with expected size. The recombinant laccase was purified from the intracellular fraction of *E.coli* by Ni-Bestarose fast flow column and HiTrap Q-HP column chromatographies. The elutions of Ni-Bestarose fast flow column by 50 mM imidazole and by 200 mM imidazole showed higher concentration of Lacc, were combined for further purification with HiTrap Q-HP column chromatographies (Figure 1b). The purified enzyme-ABTS system have an activity of 200 ± 0.53 U/mg.

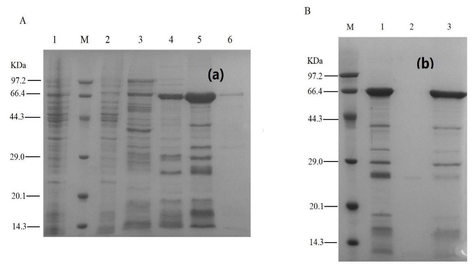


Fig.1 SDS-PAGE after laccase purification (A); SDS-PAGE after dye decolorization enzyme purification (B).

Enzymatic characterization of *B.ligniniphilus* L1 DSM 26145T laccase

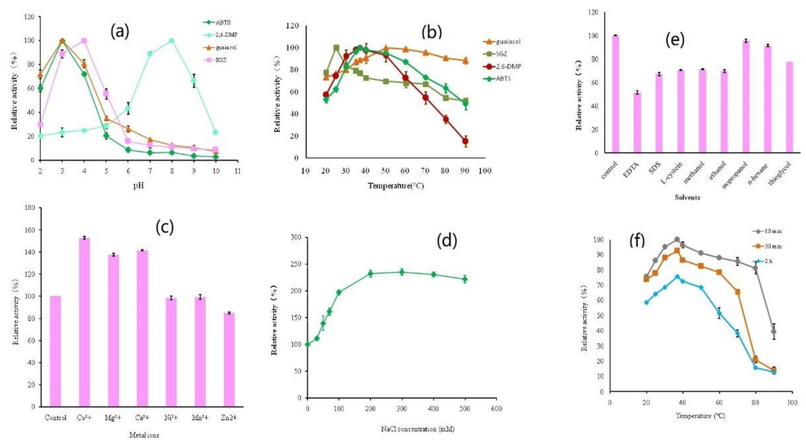


Fig.2 Catalytic properties of laccase.

The Lacc enzyme showed an activity at pH < 8 and the highest activity was observed at pH 3 (ABTS and guaiacol) and pH 4 (SGZ) with ABTS, SGZ and guaiacol as substrate (Figure 2a), and the highest activity was observed at pH 8 with 2, 6-DMP as substrate. It showed a broad range of optimum temperatures, from 25-90 ℃, exhibiting maximum activities with different substrates: 25 ℃ (SGZ), 37 ℃ (ABTS, 2, 6-DMP) and 50℃ (guaiacol), respectively (Figure 2b). Lacc has a wide range of activity and surpasses most of known bacterial laccases, exhibiting excellent tolerance to salts, organic solvents and metal ions.

Lignin discoloration

Results shown that ABTS mediator was able to increase the discoloration rate of Lacc, after 24 h incubation, the discoloration rate of AL from 60.5% was improved to 75.8% and MWL was improved from 34.2% to 64.7% (Figure 3a). Furthermore, the image of discoloring of AL and MWL also showed the color of lignin solution from brownish yellow to almost no color after 24 h incubation by LMS, and the discolorization ability of LMS was obviously stronger than Lacc ((Figure 3b). It is suggested that ABTS as mediator was able to enhance the modification of lignin by Lacc.

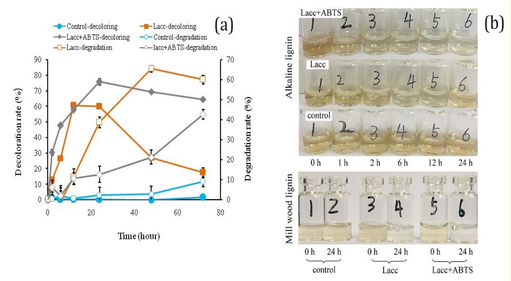


Fig.3 Lignin discoloration analysis

GC-MS analysis

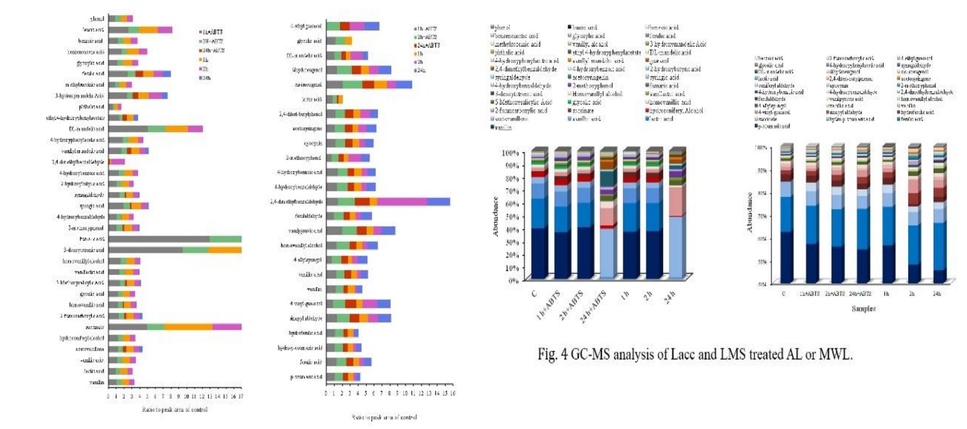


Fig. 4 GC-MS analysis of Lacc and LMS treated AL or MWL.

A total of 27 monomer aromatic compounds and 10 small molecular weight carboxylic acid compounds were identified from AL samples (Figures 4.a and c). Among them, three aromatic compounds, vanillyl alcohol, guaiacol and acetosyringenin were only present in the Lacc or LMS treated AL. And the peak areas of guaiacol and acetosyringenin in LMS samples were 2.3 and 5.4 times higher than that of Lacc samples, and vanillyl alcohol was only present in LMS samples. The above results indicated that Lacc can degrade lignin and ABTS as mediator can significantly promote the action of laccase.
In terms of abundance, vanillin has the highest proportion with 39.6% (Figure. 4b). The GC-MS data of AL treated with Lacc or LMS showed that the treatment of 1-2 h had no obvious effect on the abundance of each compound, but after 24 hours of treatment, the abundance of the major compounds greatly changed. The effect of laccase treatment on the abundance of the identified aromatic compounds and organic acids of MWL is not as significant as AL (Figure 4d).

ToF-SIMS analysis

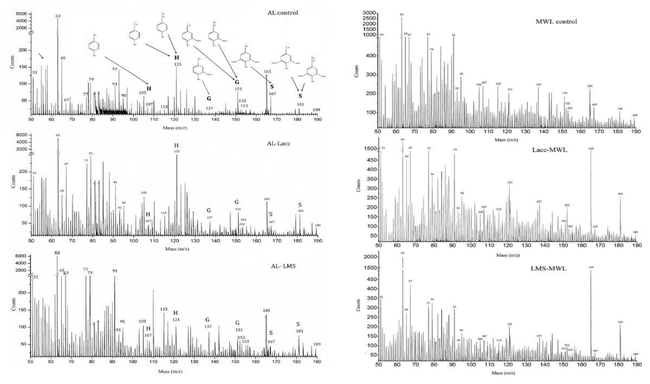


Fig.5 ToF-SIMS analysis.(a), Alkaline lignin, (b), wood mill lignin

The intensity of lignin characteristic peaks showed a significant change for AL before and after Lacc or LMS treatment (Figure 6.a, b and c). For example, the intensity of the m/z 77 and 91 peaks representing the general aromatic ring is significantly increased after treatment with Lacc or LMS. The intensity of H-lignin peak m/z 121 was enhanced after Lacc treatment but weakened after LMS treatment. For G-lignin, the peak intensity of m/z 137 increased and the peak m/z 151 has not obviously variation. The intensity of peak m/z 167 of S-lignin decreased, while the intensity of peak of m/z 181 increased. The increase of intensity of peaks of lignin units might because the repolymerization of the small molecule aromatic compound. For example, the decreasing of intensity of m/z 121 corresponds to the repolymerization of compounds belong to ρ-hydroxy-phenyl unit. The results obtained from method GC-MS also support the results of ToF-SIMS analysis.

Conclusions

(1) The laccase from *Bacillus ligniniphilus* L1 DSM 26145T was successfully expressed in *E.coli*, and exhibited relative high thermotolerance, salt and solvents tolerance. The above properties provided potential application for lignin degradation and dye discoloring.
(2) In the presence of ABTS mediator, laccase was able to effectively degrade alkaline and wood mill lignin, with the C-C bond break and side chain cleavage. However, the synergistic relationship between laccase and other lignin-degrading enzymes is still unclear and further studies are required.

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