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Mechanism of Alkaline Lignin Oxidation Using Laccase-methyl Syringate Mediator System

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Abstract

The mechanism of alkaline lignin oxidation in presence of laccase-methyl syringate (MS) mediator is discussed in terms of morphological changes that take place during exposure of the lignin to the phosphate buffer solution (pH=6.5) for 72hr at 70^{o}C. The SEM analysis of lignin before and after enzymatic treatment reveals the morphological changes explained by the interaction of the lignin surface groups with laccase-methyl syringate system. The BET analysis confirms that this interaction causes the change in the surface area from 2.75 to 5.50cm^2/g. The corresponding pore-size distribution in lignin sample treated with laccase-methyl syringate is much broader in comparison to the untreated lignin and the pores within 25-150nm range are detected as a result of the BJH analysis. The electrochemical study of lignin, lignin with laccase, and lignin with laccase in presence of the mediator in the buffer solution has been performed in the potential range from -0.3 to +1.0V vs. Ag, AgCl, Cl$^-$ reference electrode. The cyclic voltammetry confirms reversible oxidation-reduction behavior of the methyl syringate natural mediator in anaerobic and aerobic environment. Specifically in anaerobic conditions three oxidation anodic peaks (0.265V, 0.474V and 0.884V) and two reduction peaks (0.421V and 0.103V) were detected out of which the oxidation peak at 0.474V was assigned to the formation of MS· radical. In aerobic conditions the methyl syringate demonstrates two oxidation peaks (0.473V and 0.812V) and two reduction peaks (0.410V and 0.135V). The mechanism of MS radical stability in oxygen vs. anaerobic environment is proposed based on formation of MS· radicals.

Key words: methyl syringate, laccase, lignin electrooxidation, laccase-mediator system

1. Introduction

Lignin is the second most abundant natural polymer following cellulose, but the commercial use of lignin which is mostly produced by paper industry is only 2%. The rest of the available lignin is usually burned for providing the heat (Chapple et al., 2007) that does not satisfy the need for effective power generation. Different ways of lignin recycling by using catalysts and ionic liquids are reflected in numerous publications (Deepa & Dhepe, 2015., Nanayakkara et al., 2014) however these approaches are often expensive and do not meet the requirements for sustainable lignin recycling. One of the environmentally friendly approaches is to use naturally existing enzymes that are capable of degrading lignin and biomass (Christopher et al., 2014).

Lignin is recalcitrant to degradation because of the complex cross-linked network structure. The white-rot fungi have the efficiency and selectivity to biodegrade the lignin due to production of the lignolytic enzymes (Niladevi, 2009) which are categorized to peroxidases and laccases (Hori et al., 2014). Laccase (oxygen oxidoreductase) is a multicopper oxidase that has an ability to participate in the biodegradation of lignin (Higuchi, 2004; Hoopes & Dean, 2004). Due to the low electrochemical reduction potential, laccase can only oxidize the phenolic lignin moiety (<20% of total lignin) and not the non-phenolic aromatic structure (80% of total lignin) (Camarero et al., 1994). Moreover, lignin's microporous network is not readily accessible for laccase molecules due to their large size (~5-6 nm) (Gascón et al., 2014) and further decreases the overall oxidation efficiency.

The mediator is a small molecule which could be oxidized by laccase and reduced by the substrate. Mediators with low molecular weight have a redox potential higher than 0.9V and can serve as the electron carriers. On the other hand,

mediators, as small organic molecules, can enter the pores of lignin and perform a role of electron carriers between the laccase and the lignin. The mediator in an oxidized state has high redox potential (Li et al., 1999) and the ability to oxidize the non-phenolic moiety of lignin. To facilitate the oxidation of lignin by an enzyme, the natural mediators (e.g. 3,5-dimethoxy-4-hydroxybenzaldehyde and methyl syringate (Díaz-González et al., 2011)) and synthetically produced mediators (e.g. ABTS (Bourbonnais & Paice, 1990) or HBT (Call, 1994)) have been proposed. Compared to artificial mediators, the natural mediators are less toxic and more economically feasible. Mediators can be oxidized by the laccase and reduced by the substrate. The optimal mediator is able to keep the cyclic and reversible redox reaction going by reducing to the initial form. However, the mediator should not inhibit the laccase activity during the process of alkali lignin degradation.

Natural phenolic mediators, such as methyl syringate (Wells, 2006) from *Myceliophthora thermophile* (Alejandro Rico1, 2013) can be oxidized by laccase and enhances the oxidization of non-phenolic moiety of lignin and lignin model compounds. As an example, methyl syringate (Díaz-González et al., 2011) and 4-hydroxy-3, 5 dimethoxyacetophenone act as enhancers with laccase from *Trametes villosa* in electrochemical oxidation of kraft and flax lignin, respectively (González Arzola et al., 2009).

The goal of the present study is to investigate the mechanism of alkali lignin oxidation in presence of methyl syringate in the laccase mediator system (LMS) in terms of morphological changes and electrochemical behavior. The electrochemical comparison of the laccase, mediator, and the laccase-mediator system behavior toward degradation of lignin directly deposited on the surface of the rotating glassy carbon electrode in aerobic and anaerobic environment is presented for the first time.

2. Materials and Methods

2.1 Materials

Methyl syringate (MS) mediator and laccase were provided by Novozyme Corp. (Denmark). Disodium hydrogen phosphate (Na_2HPO_4), monosodium phosphate (NaH_2PO_4), 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and alkali lignin were purchased from Sigma-Aldrich (US). The laccase activity was measured as an initial velocity during oxidation of 1.6 mM ABTS from Roche to its cation radical (ε_{420} =36000 $M^{-1}.cm^{-1}$) in 0.2M Na_2HPO_4-NaH_2PO_4 buffer solution (pH 6.5) at 30°C. The laccase activity of the enzyme was 326 U/mL. One activity unit (U) was defined as the amount of enzyme transforming 1 μmol of ABTS per min.

2.2 Morphological Study

The lignin samples before and after LMS treatment by LMS were tested with a Zeiss Supra 40VP variable-pressure field emission SEM. The scanning electron micrographs were recorded under an electron beam acceleration of 1 keV at a 5 mm working distance using an in-lens detector. The surface morphology of the materials was characterized using multi point N_2-physisorption isotherms at 77K by means of an automated Quantachrome Nova2200e. The degassing of the catalysts was conducted at 80^{o}C in N_2 overnight prior to the analysis.

2.3 Electrochemical Evaluation

Cyclic voltammetry (CV) - experiments were performed with a Pine bi-potentiostat (AFCBP1, Pine Research Instrumentation, USA) attached to an analytical three-electrode configuration (RDE0018) from Princeton Applied Research. All measurements were carried out in a 100-mL cell at room temperature. A glassy carbon electrode (GCE) with deposited lignin film was used as a working electrode. A platinum wire was used as a counter-electrode and a silver-silver chloride electrode (Ag, AgCl, Cl^-) served as a reference electrode. Before each experiment, the surface of the glassy carbon electrode was polished on a diamond-polishing pad followed by washing with distilled water. To prepare a working electrode, a 20 μL of alkali lignin dispersed in phosphate buffer solution (0.1 mg/mL) was dropped onto the polished surface of the GC working electrode and allowed to dry for 15 min at room temperature. All CVs were recorded in 0.2 M phosphate buffer solution Na_2HPO_4-NaH_2PO_4 (pH=6.5) in absence or presence of 1.48 mM methyl syringate. The electrode potential was scanned from -0.3 to 1.0 V at different scan rates of 50 mV/s, 100 mV/s and 200 mV/s. Glassy carbon electrode (GCE) in aqueous solutions is considered to be an inert electrode for hydronium ion reduction (Gattrell & Kirk, 1990; Calderon et al., 2013).

3. Results and Discussion

3.1 Morphological Study of Lignin Oxidation in Presence of the Laccase -methyl Syringate System

The SEM analysis reveals the changes in the surface morphology associated with alkali lignin before and after the LMS treatment (Figure 1). The surface morphology of the lignin (Figure 1a-d) improves after the LMS treatment (Figure 1e-h). Specifically, there are more pores present on the surface of the lignin compared to the non-treated lignin (Hamed, 2013). This observation is confirmed by the BET analysis (Figure 2).

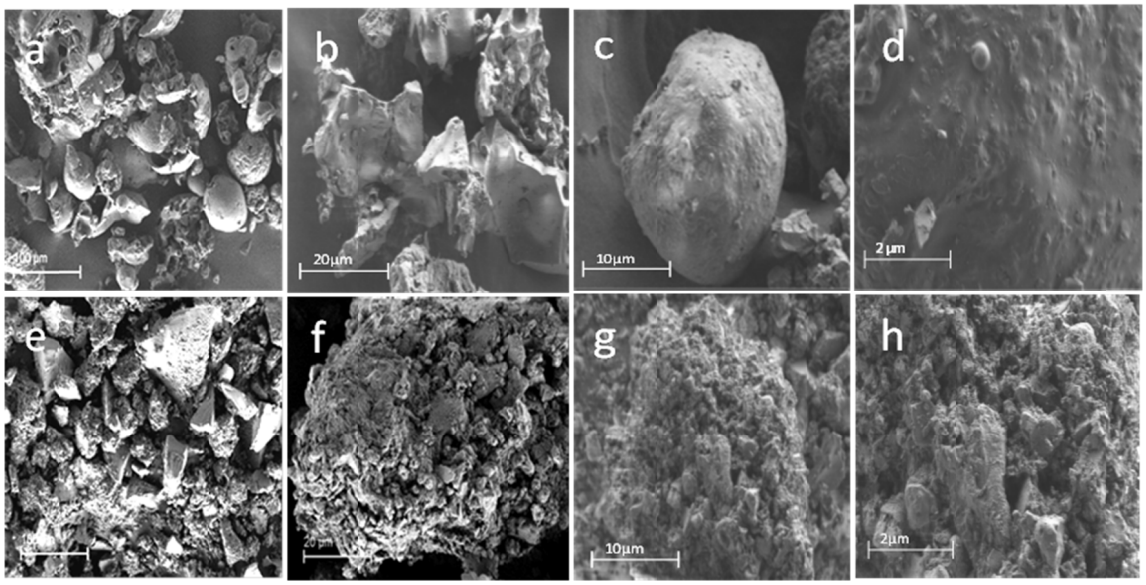


Figure 1. SEM of alkali lignin before (a-d) and after (e-h) treatment with LMS.

The Brunauer–Emmett–Teller (BET) analysis of the alkali lignin has been conducted before and after contact with the laccase-mediator system (5% *Myceliophthora Thermophila* laccase and 5% methyl syringate mediator) in oxygen-rich environment at T=70℃for 72 hrs in Na_2HPO_4-NaH_2PO_4, pH=6.5. The buffer solution contained 5% *Myceliophthora Thermophila* laccase and 5% methyl syringate mediator. Multi point BET isotherms and Barrett–Joyner–Halenda (BJH) pore size distribution in the range between 2-200 nm were presented (Figure 2 a, b). The N_2 adsorption-desorption isotherms (Figure 2a) show a hysteresis due to nitrogen capillary-condensation phenomenon which is typical to mesoporous materials. The Specific Surface Areas (SSAs) and pore volumes corresponding to partial pressures of p/p_o=0.30 and 0.99, respectively were improved up to 2 times after the LMS treatment. Pore-Size-Distribution (PSD) comparison of the alkali lignin (Figure 2b) indicates that the pore-volume was improved due to formation of new mesopores after the LMS degradation. The results indicate that the chosen LMS is active and participates in lignin degradation. This observation is in good agreement with SEM analysis and confirms that at the chosen conditions the LMS is actively involved in the lignin degradation process.

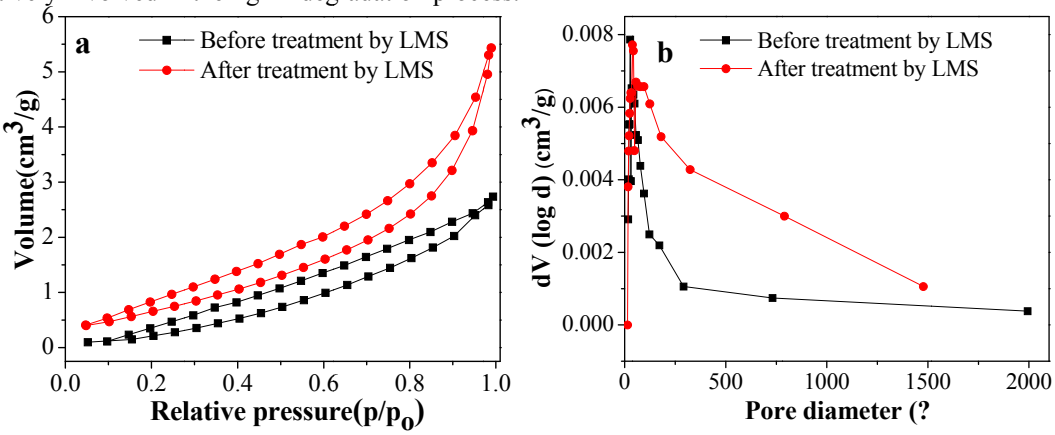


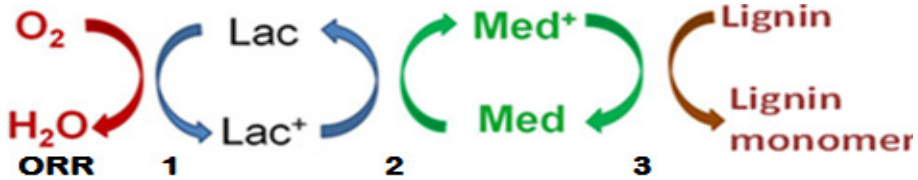
Figure 2. Comparison of the nitrogen adsorption-desorption isotherms (77K) of the alkali lignin (a) and the pore size distribution before and after LMS treatment (b).

3.2 Electrochemical Study of the Lignin-LMS System

3.2.1 Alkali Lignin in Presence of Laccase

The lignin oxidation in presence of LMS demonstrates three different redox electrochemical reactions coupled in the process of lignin decomposition (Figure 3). The presented mechanism indicates that the enzyme alone (Lac^+) is not capable of complete lignin oxidation. As expected, in presence of atmospheric oxygen, an oxygen reduction reaction (ORR) takes place and the atmospheric oxygen participates in the oxidation of laccase (Figure 3, step 1) (Wong, 2009). The laccase has an ability to participate in a redox process on the surface of the reducing substrate involving the four-electron transfer from substrate to each molecular oxygen (Wong, 2009). The laccase can oxidize the mediator to

the mediator radical which has high redox potential. (Figure 3, step 2). However, the mediator at a high redox potential (>0.9 V) is capable of oxidizing the lignin non-phenolic groups by itself (Figure 3, step 3). Therefore the laccase can oxidize the mediator as an electron transfer agent (Figure 3, step 2) with a redox potential normally higher than 0.9 V. Then mediator, in turn, can oxidize lignin (Figure 3, step 3). It is known that in the laccase- mediator systems the mediators form stable free ion radicals performing as oxidizing compounds (Morozova et al., 2007). The charged mediator species (Med^+) can diffuse away from the enzyme easily penetrating the lignocellulose matrix and initiating the process of lignin oxidation and depolymerization.



To evaluate the electrochemical behavior of the alkali lignin in presence of the laccase, the CV plots of the laccase in nitrogen (imitating anaerobic conditions) and oxygen have been measured (Figure 4a-b). Fifteen cycles were performed for each test to ensure stabilization of the cyclic voltammetry data. For the laccase alone, the redox transformation in the potential range between -0.3 and 1.0 V vs. Ag/AgCl, Cl^- electrode has not been detected at 50 mV/s scan rate indicating that the laccase without lignin does not have a noticeable redox activity. In case of lignin, the CV at 50mV/s shows an oxidation peak E_{pa} at 0.249V and the corresponding reduction peak E_{pc} at 0.035V in nitrogen. One of the explanations for the observed lignin electrochemical activity detected in anaerobic environment could be related to the molecular oxygen within the pores of lignin. However, considering that this peak remains constant after fifteen cycles an alternative explanation can be given. Specifically, this activity can be due to the electrochemically active phenolic groups on the lignin surface. After addition of laccase to the phosphate buffer solution in presence of lignin on the RDE surface, the oxidation and reduction peaks of lignin shift to the lower potentials of 0.245V and 0.023V, respectively (Figure 4a). Furthermore, the corresponding increase in current density indicates that there is an interaction between the active groups on the surface of lignin and the laccase. The capacitive behavior of the alkali lignin on the surface of glassy carbon in presence of laccase increases, which is in correlation with the SEM data (Figure 1).

The laccase oxidation by molecular oxygen is the first step of lignin decomposition process (Figure 3) (Morozova et al., 2007). Thus, considering our assumption that the electrochemically active groups on the lignin surface are responsible for the redox reversible behavior, similar experiments were performed in oxygen atmosphere. The CVs in anaerobic conditions were compared to the results acquired in oxygen. One anodic and one cathodic faradaic peak at 0.190V to 0.250V, respectively, corresponding to a reversible redox behavior of the phenolic groups on the lignin surface is visible in Figure 4b. The anodic peak is a result of lignin oxidation of by the laccase in the oxidized state (Lac^+) which is involved in the oxidation of phenolic groups. However, there is almost no difference between the CV plots in N_2 and O_2 for laccase that proves our concept that in phosphate buffer solution in the range of the applied voltages the peaks are attributed to the phenolic groups of lignin. As expected, there is no difference in the electrochemical behavior of the laccase alone in N_2 or O_2 (Figure 4a, b) since the laccase does not have electrochemically active groups in this potential range. Considering the lignin alone there is a shift in the oxidation peak voltage of about 59mV in N_2 (0.249V) vs. O_2 (0.190V) due to the presence of phenolic groups on the lignin surface. When the laccase is added to lignin, the difference between N_2 and O_2 becomes more visible: the corresponding shift increases to 85mV in N_2. Furthermore, in the case of N_2 the redox currents of the system including both lignin and laccase in comparison to the pure lignin in absence of laccase are higher which is in correlation with a positive effect of laccase in the lignin oxidation.

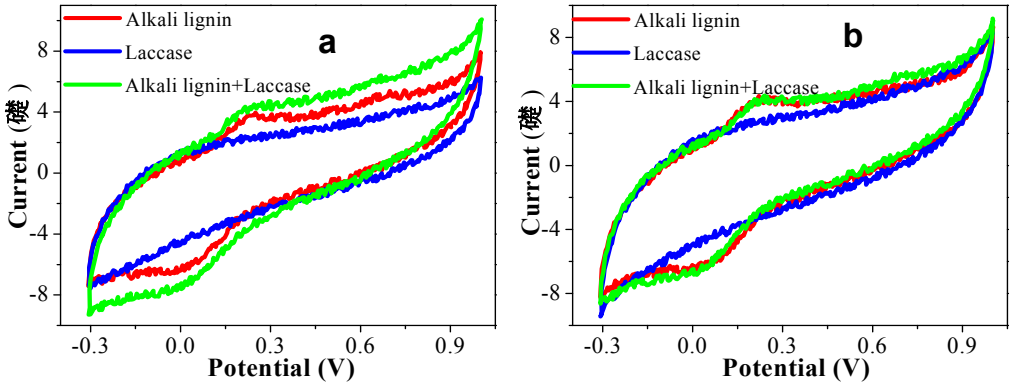


Figure 4. Cyclic voltammograms of alkali lignin, laccase (326 U/mL), and alkali lignin-laccase system in 0.2 M Na_2HPO_4-NaH_2PO_4 (pH=6.5) buffer solution at 50 mV/s in N_2 (a) and O_2 (b) atmosphere.

3.2.2 Alkali Lignin in Presence of the Methyl Syringate Mediator

To determine the reactivity of lignin surface groups with methyl syringate in the oxidized state, (Figure 5) the cyclic voltammograms of MS alone and with alkali lignin in nitrogen atmosphere were performed. The MS alone demonstrates reversible redox behavior (Figure 5a) with three oxidation anodic peaks (E_{pa}) at 0.265V, 0.474V and 0.884V and only two reduction peaks (E_{pc}) at 0.421V and 0.103V. The oxidation peak at 0.474V can be assigned to the formation of (MS·) radical involved in the redox reversible process.

The oxidation peak at 0.265V is irreversible and can be assumed as resulting from the byproduct ($MS^{\cdot+}$) formed during the mediator oxidization. During MS oxidation, the radical cation ($MS^{\cdot+}$) formed from methyl syringate molecule is produced and can further dissociate forming the radical $MS^{\cdot}$:

$$MS^{\cdot+} \rightarrow MS^{\cdot} + H^{+} \quad (1)$$

The electrochemical oxidation of MS and alkali lignin results in decrease of the MS oxidation peak. The peak at 0.884V is the result of the oxidation of the methyl syringate radical (Díaz González et al., 2009).

The difference between the oxidation and the reduction peaks (E_{pa}-E_{pc}) is 0.473 V and the ratio of the peak currents equals to i_{pa}/i_{pc} =3.40. This result indicates that the MS· radicals produced in the process of the electrochemical oxidation of MS are not stable and decay to a non-reducible compounds in the nitrogen atmosphere.

Addition of lignin to the MS solution in N_2 (Figure 5a) significantly changes the current density and the shape of the CV plot. In comparison to pure lignin the current increases at high potentials (> 0.8V vs. Ag, AgCl, Cl^{-}). The decrease of the current peak at 0.506 V for a mixture of lignin with MS in comparison to MS alone can be attributed to the partial loss of the redox activity of the MS in presence of lignin and the MS interaction with the lignin surface groups. Furthermore, the polymerization of the phenolic oxidation products on the electrode surface can take place due to the formation of a polymeric passivating layer (Aracri et al., 2013). After the methyl syringate is added, the oxidation of the non-phenolic groups of lignin corresponding to a weak cathodic peak appears far away from the oxidation peak (Aracri et al., 2013). This effect indicates partial blocking of MS electron transfer to and from the electrode, produced by lignin deposition on the electrode surface. Possible explanation could be related to the relatively high Lignin: MS ratio that was 350:1 significantly exceeding the previous values of 10:1 (Aracri et al., 2013). Furthermore the ratio of 10:1 considered earlier for lignin model compound (that was present in solution, rather than on the GC electrode surface) can impose mass-transport limitations and decrease the catalytic efficiency (CE) (Aracri et al., 2013).

Similar to nitrogen atmosphere, in the oxygen environment the cyclic voltammetry of the MS shows a reversible redox behavior. The values of the two oxidation MS anodic peaks (E_{pa}) at 0.473V and 0.812V, and the two reduction cathodic peaks (E_{pc}) at 0.410V and 0.135V (Figure 5b) are close to those obtained in N_2. The current density for each of the oxidation anodic currents increases twice due to the presence of oxygen compared to the anodic currents in nitrogen.

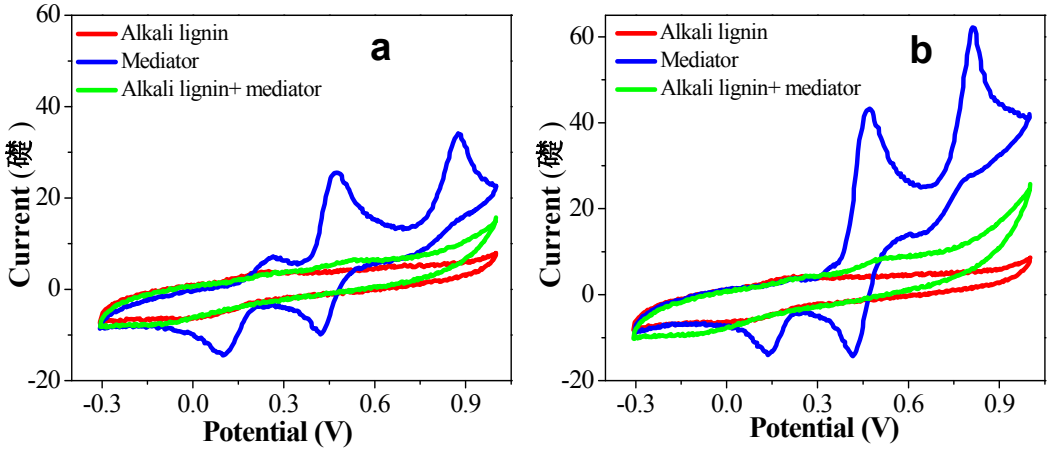


Figure 5. Cyclic voltammograms of the MS mediator (1.48 mM) and MS with 0.1 mg/mL alkali lignin on RDE surface in 0.2 M Na_2HPO_4-NaH_2PO_4 (pH=6.5) at 50mV/s scan rate in N_2 (a) and O_2 (b) atmosphere.

3.2.3 Cyclic Voltammetry of Laccase-methyl Syringate System

The CV plots of laccase in N_2 (Figure 6a) demonstrate that laccase does not show the electrochemical activity in the phosphate buffer solution. However, in absence of laccase the mediator demonstrates a reversible oxidation-reduction behavior. After the laccase is added to the MS mediator, the two oxidation peaks (0.474V, 0.884V) of mediator shift to higher potentials and two reduction peaks (0.421V, 0.103V) shift to lower potentials. Moreover, the currents of the two

oxidation peaks increase, which indicates that the laccase has ability to oxidize the mediator even in nitrogen atmosphere.

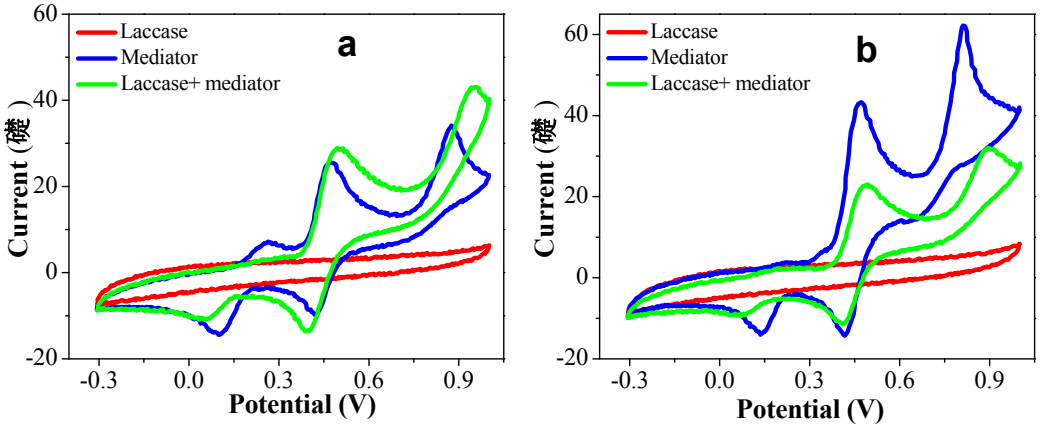


Figure 6. Cyclic voltammograms of laccase (326 U/mL), mediator (1.48mM) and laccase -mediator (1.48mM) in 0.2 M Na_2HPO_4-NaH_2PO_4 (pH=6.5) at 50mV/s scan rate in N_2 (a) and O_2 (b) atmosphere.

However, there is a significant difference between the CV plots in N_2 and O_2 indicating that the peak current densities are higher in O_2 than in N_2. Furthermore, the voltage shifts take place. Specifically, in O_2 the CV plot for the laccase-mediator system (Figure 6b) shows the first peak at lower potential (0.491V). However, the second oxidation peak for laccase-mediator is shifted to the lower voltage (0.898V) due to the oxidation of the mediator promoted by the ORR in presence of oxygen.

Addition of the laccase to the mediator (Figure 6b) causes significant current drop due to low stability of MS radicals in oxygen atmosphere and deposition of the laccase-oxidized species on the surface of the rotating disk electrode (Coote & Henry, 2005). These species decrease the mass transport limitations for the mediator molecules, thus minimizing the synergistic effect between the laccase and the mediator. Additional CV data for the mediator, laccase, and the mediator with laccase at different rotation speeds (0-900rpm) and scan rates (50-200mV/sec) generated in this study indicate that in all the cases the mediator redox currents in comparison to the laccase with mediator system are higher in oxygen vs. nitrogen.

3.2.4 Alkali Lignin in Presence of Laccase-methyl Syringate System

The comparison of the CV plots of lignin-mediator system and lignin-laccase-mediator system in N_2 atmosphere show that there is a little shift between two of the oxidation peaks of lignin-mediator (0.536V) and lignin-laccase-mediator (0.586V). The first oxidation peak of lignin-laccase system at 0.249V has the highest current as a result of oxidation of phenolic group of lignin by laccase (Figure 7a). The oxidation of the non-phenolic groups appears at 0.506V. The oxidation peak currents at 0.506V increase after the laccase is added into the lignin-mediator system. This can be explained by the high stability of the MS· radical that has enough time to react with the non-phenolic groups on the surface of lignin.

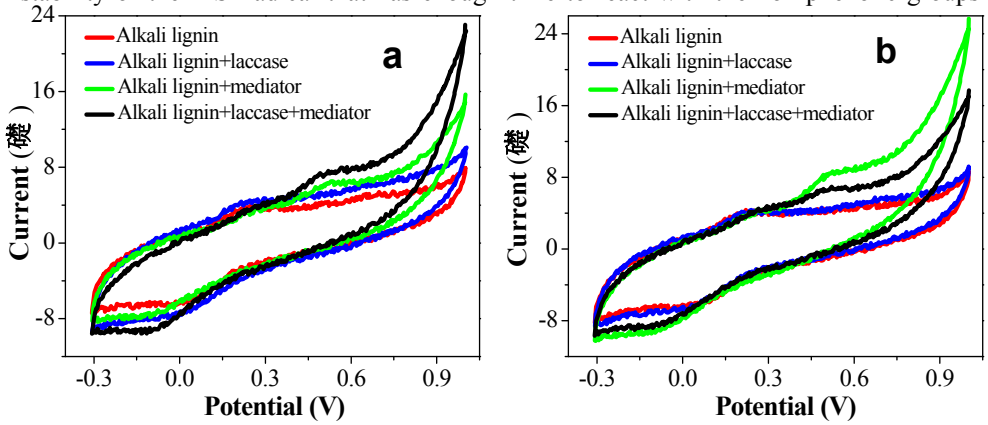


Figure 7. Cyclic voltammograms of alkali lignin (0.1 mg/mL), alkali lignin-laccase (326 U/mL), alkali lignin-mediator (1.48mM) and alkali lignin -laccase -mediator in 0.2 M Na_2HPO_4-NaH_2PO_4 (pH=6.5) at 50mV/s scan rate in N_2 (a) and O_2 (b) atmosphere.

In the oxygen atmosphere, the oxidation peak of lignin-mediator system at 0.525V shifts to 0.521V when the laccase is added. According to the mechanism (Figure 3), more mediator radicals could be produced in oxygen atmosphere than in the nitrogen atmosphere. However, depending on CV analysis, the stability of the radicals has higher effect on the CV potential than the number of produced radicals in oxidation of the non-phenolic groups of lignin (Figure 7b). The reason of the current decrease can be explained by the fact that the radicals are not stable in oxygen condition and do not have enough time to oxidize the non-phenolic groups of lignin.

4. Conclusions

The performed morphological studies of the alkali lignin in presence of laccase-mediator system indicate that the surface of alkali lignin is modified resulting in significant surface modification. The BET demonstrates that the specific surface area and pore volume of the alkali lignin increases up to two times after the LMS treatment. The reason for the improvement of the pore volume is in formation of the new mesopores, which indicates that the proposed LMS system can actively participate in the lignin degradation. The cyclic voltammetry has been used as a simple and powerful tool to predict the efficiency of the laccase mediator systems in the lignin oxidative process under the specified experimental conditions. In presence of MS, an increase in lignin oxidative behavior at higher potentials suggests that the oxidation of the non-phenolic structures in lignin takes place. Based on the electrochemical study, an assumption was made that the stability of MS· radicals is more important than the number of the MS· mediator radicals.

Acknowledgements

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References

A. Wells, M. T. A.T. E. (2006). Green oxidations with laccase–mediator systems, *Biochem. Soc. Trans., 34*, 304–308.

Alejandro, R. J. R., Jose, C. D. R., Angel, T. M., & Ana, G. (2013). Pretreatment with laccase and a phenolic mediator degrades lignin and enhances saccharification of Eucalyptus feedstock, *Biotechnol Biofuels., 7*.

Aracri, E., Tzanov, T., & Vidal, T. (2013). Use of Cyclic Voltammetry as an Effective Tool for Selecting Efficient Enhancers for Oxidative Bioprocesses: Importance of pH, *Ind Eng Chem Res., 52***,** 1455-1463. http://dx.doi.org/10.1021/ie3027586.

Bourbonnais, R., & Paice, M. G. (1990). Oxidation of non-phenolic substrates: An expanded role for laccase in lignin biodegradation, *FEBS Letters., 267***,** 99-102. http://dx.doi.org/10.1016/0014-5793(90)80298-W.

Calderon, E. H., Dangate, M., Manfredi, N., Abbotto, A., Salamone, M. M., Ruffo, R., & Mari, C. M. (2013). Electrochemical and Spectroelectrochemical Properties of a New Donor¨CAcceptor Polymer Containing 3,4-Dialkoxythiophene and 2,1,3-Benzothiadiazole Units, *Polymers*.. http://dx.doi.org/10.3390/polym5031068.

Call, H. P. (1994). Process for modifying, breaking down or bleaching lignin materials containing lignin or like substances, *World patent application* WO 94/29510.

Camarero, S., Galletti, G. C., & Martínez, A. T. (1994). Preferential degradation of phenolic lignin units by two white rot fungi, *Appl. Environ. Microbiol., 60*, 4509-4516.

Chapple, C., Ladisch, M., & Meilan, R. (2007). Loosening lignin's grip on biofuel production, *Nat Biotech., 25*, 746-748.

Christopher, L. P., Yao, B., & Ji, Y. (2014). Lignin biodegradation with laccase-mediator systems, *Front. Chem. Eng.Res., 2*, 1-13. http://dx.doi.org/10.3389/fenrg.2014.00012.

Coote, M. L., & Henry, D. J. (2005). Effect of Substituents on Radical Stability in Reversible Addition Fragmentation Chain Transfer Polymerization: An ab Initio Study, *Macromolecules., 38*, 1415-1433. http://dx.doi.org/10.1021/ma047814a.

Deepa, A. K., & Dhepe, P. L. (2015). Lignin Depolymerization into Aromatic Monomers over Solid Acid Catalysts, *ACS Catal., 5*, 365-379. http://dx.doi.org/10.1021/cs501371q.

Diaz-Gonzalez, M., Vidal, T., & Tzanov, T. (2011). Phenolic compounds as enhancers in enzymatic and electrochemical oxidation of veratryl alcohol and lignins, *Appl Microbiol Biotechnol., 89*, 1693-1700. http://dx.doi.org/10.1007/s00253-010-3007-3.

Diaz Gonzalez, M., Vidal, T., & Tzanov, T. (2009). Electrochemical Study of Phenolic Compounds as Enhancers in Laccase-Catalyzed Oxidative Reactions, *Electroanal., 21***,** 2249-2257. http://dx.doi.org/10.1002/elan.200904678.

Gascon, V., Diaz, I., Márquez-Alvarez, C., & Blanco, R. (2014). Mesoporous Silicas with Tunable Morphology for the Immobilization of Laccase, *Molecules., 19*, 7057.

Gattrell, M., & Kirk, D. W. (1990). The electrochemical oxidation of aqueous phenol at a glassy carbon electrode, *Can. J. Chem. Eng., 68*, 997-1003. http://dx.doi.org/10.1002/cjce.5450680615.

González. A. K., Arévalo, M. C., & Falcón, M. A. (2009). Catalytic efficiency of natural and synthetic compounds used as laccase-mediators in oxidising veratryl alcohol and a kraft lignin, estimated by electrochemical analysis, *Electrochim. Acta., 54*, 2621-2629. http://dx.doi.org/10.1016/j.electacta.2008.10.059.

Hamed, S. A. M. (2013). In-vitro studies on wood degradation in soil by soft-rot fungi: Aspergillus niger and Penicillium chrysogenum, *Int. Biodeterior. Biodegradation., 78*, 98-102 http://dx.doi.org/10.1016/j.ibiod.2012.12.013.

Higuchi, T. (2004). Microbial degradation of lignin: Role of lignin peroxidase, manganese peroxidase, and laccase, *Proc. Jpn. Acad., Series. B, 80*, 204-214.

Hoopes, J. T., & Dean, J. F. D. (2004). Ferroxidase activity in a laccase-like multicopper oxidase from Liriodendron tulipifera, *Plant Physiol Biochem., 42*, 27-33. http://dx.doi.org/10.1016/j.plaphy.2003.10.011.

Hori, C., Gaskell, J., Igarashi, K., Kersten, P., Mozuch, M., Samejima, M., & Cullen, D. (2014). Temporal Alterations in the Secretome of the Selective Ligninolytic Fungus Ceriporiopsis subvermispora during Growth on Aspen Wood Reveal This Organism's Strategy for Degrading Lignocellulose, *Appl Environ Microbiol., 80*, 2062-2070. http://dx.doi.org/10.1128/aem.03652-13.

Li, K., Xu, F., & Eriksson, K. E. L. (1999). Comparison of Fungal Laccases and Redox Mediators in Oxidation of a Nonphenolic Lignin Model Compound, *Appl Environ Microbiol., 65*, 2654-2660.

Morozova, O. V., Shumakovich, G. P., Shleev, S. V., & Yaropolov, Y. I. (2007). Laccase-mediator systems and their applications: A review, *Appl. Biochem. Microbiol., 43*, 523-535. http://dx.doi.org/10.1134/S0003683807050055.

Nanayakkara, S., Patti, A. F., & Saito, K. (2014). Lignin Depolymerization with Phenol via Redistribution Mechanism in Ionic Liquids, *ACS Sustain Chem Eng., 2*, 2159-2164. http://dx.doi.org/10.1021/sc5003424.

Niladevi, K. N. (2009). "Ligninolytic Enzymes," in *Biotechnology for Agro-Industrial Residues Utilisation,* eds. P. Singh Nee' Nigam & A. Pandey, *Springer Netherlands*., 397-414.

Wong, D. S. (2009). Structure and Action Mechanism of Ligninolytic Enzymes, *Appl Biochem Biotechnol., 157*, 174-209. http://dx.doi.org/10.1007/s12010-008-8279-z.