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Research article

Biodegrading Lignocellulosic Agricultural Waste Using *Phanerochaete chrysosporium* and Electrical Current Stimulation

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Abstract: White-rot fungi (WRF), such as *Phanerochaete chrysosporium*, play a significant role in the lignin degradation (LD) of biomass, an essential process in the carbon recycling of terrestrial ecosystems. However, the rapid development of the agroindustry has imposed a daunting task on biomass waste management. One green initiative focuses on enhancing the bioremediation of lignin since it forms a resistant barrier to chemical and biological LD. This work demonstrated that electric current stimulation (ECS) can markedly enhance LD by *P. chrysosporium*. Palm oil empty fruit bunches (EFBs) were utilized as a lignin-rich substrate for *P. chrysosporium*. These were placed in a 250-ml enclosure filled with unbuffered potato dextrose broth (PDB) as the electrolyte. The ECS was supplied in situ in two ways: (1) by inserting a zinc anode/air electrode redox couple into the enclosure to produce a self-sustaining discharge current (DC), and (2) by connecting the enclosure to an external current (EC). The lignin content (LC) of the EFBs was assessed after 30 days of exposure to fungal microbes in an uncontrolled environment. The fungal LD rate was highest at 3 mA and even doubled under the influence of the EC, enhancing the lignin removal by 74.6%. The proposed method is much simpler and cheaper than the electrocatalytic reactions produced by the electro-Fenton method.

Keywords: Lignin degradation; microbial zinc/air cell; electric current stimulation; white rot fungi

1. Introduction

The agroindustry generates a huge quantity of biomass wastes and residues. Without serious efforts and mitigation measures, this will ultimately lead to significant environmental pollution. Growing environmental concerns have pushed the industry's sustainable development to center stage. Bioremediation of biotransformation has been identified as a potential secondary technology for converting biomass wastes into valuable byproducts or their reproduction into environmentally benign byproducts. Empty fruit bunches (EFBs) are a solid biomass the palm oil industry produces. On average, 50-70 tonnes of biomass are produced from a hectare of oil palm plantation. Empty fruit bunches (EFBs) contribute 20-23% of solid waste [1]. Unfortunately, most of this biomass is either incinerated, disposed of as waste in landfills, or dumped on plantation grounds

as mulch [1]. Although these disposal methods are cost-effective, they can lead to greenhouse gas emissions and create homes for pests [2].

Using white-rot basidiomycetes for wood's lignin degradation (LD) is a central step of carbon recycling in terrestrial ecosystems. As such, they have been extensively studied for the bioremediation of wastes and residues from the agroindustry. White-rot fungi (WRF), such as *Phanerochaete chrysosporium*, *Pleurotus ostreatus, Trametes versicolor*, and *Xylobolus frustulatum*, have a unique ability to break down wood lignin [3,4]. Lignin is a recalcitrant material that forms the protective structural matrix surrounding the hemicellulose and cellulose within cell walls [5,6]. It is not only abundant in nature but is also a potential source of renewable chemicals and energy. However, the main challenge lies in its intrinsic mechanical and chemical resistance. *P. chrysosporium* has been widely researched for this purpose, given its ability to produce a more complete LD enzyme complex than most other strains [7].

Earlier, we reported on a self-sustaining microbial zinc/air cell (MZAC) employing *P. chrysosporium* fed with oil palm EFBs [8]. This lignocellulosic biomass was mainly composed of three main components: cellulose (44.2%), hemicellulose (33.5%), and lignin (20.4%) [9]. The anodic zinc oxidation was believed to provide a continuous supply of free electrons for the LD of the EFBs. It was thought that such a condition would most likely alter the metabolism pathways of the microbes and eventually influence the LD rates of the biomass. Therefore, this work assessed *P. chrysosporium* and electric current stimulation (ECS) for LD in EFBs. First, a discharge current was generated by the electrochemical reactions of the MZAC fed with the fungus-cultivated EFBs. Second, an external current was used to pass an ECS through the enclosure containing the fungus-cultivated EFBs.

Microbes' metabolic activities, bacteria, and fungi have been reported to be strongly influenced by an ECS. This is of particular interest for enhancing the bioremediation of environmental pollutants. Vasileva et al. observed that the symbiotic *Bradyrhizobium japonicum* bacterium's phenolic degradation capability was enhanced by applying an electric field [10]. Since EFBs consist of lignin with a highly-branched phenolic polymer, it would also be interesting to observe the influence of an applied electric field on LD. Shen et al. used an advanced electro-Fenton method to promote LD using the WRF, *T. versicolor* [4]. In the electro-Fenton method, the electro-oxidation of the iron-based composite cathode generates hydroxyl radicals. These strong oxidants attack various functional groups in lignin, thereby accelerating the LD using *T. versicolor* [4]. The present study proposed a much simpler method of enhancing LD using another WRF, *P. chrysosporium*, and a small ECS.

2. Materials and Methods

2.1 Selecting the raw materials and microorganisms

Empty fruit bunches (EFBs) were collected from a palm oil plantation. The raw EFBs were manually shredded and sieved into small sizes of around 0.5 cm. These were soaked for 24 hours, rinsed with distilled water, and dried under ultraviolet (UV) light. The WRF, *P. chrysosporium*, was used as the organic waste-degrader and cathodic catalyst source in the MZAC. *P. chrysosporium* secretes several ligninolytic enzymes, such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase. Of these three enzymes, laccase plays a crucial role in MZACs due to its ability to reduce oxygen (O_2) to water (H_2O). *P. chrysosporium* was first cultivated in potato dextrose agar (PDA) for six days and was then transferred into a potato dextrose broth (PDB) for re-cultivation for 14 days. The specimen was filtered out from the PDB and dried until its moisture content was < 15%.

2.2 Preparing the microbial zinc/air cell (MZAC)

The cell was a membrane-less, single-chamber cylindrical jar with a capacity of 250 ml that was filled with 24 g/L of unbuffered PDB electrolyte. A zinc anode strip (30 x 30 mm) was paired with a commercial E4/E4A-EFL air cathode of the same dimensions. The fungus-cultivated EFBs were prepared from 5 g of dried *P. chrysosporium* and 2 g of oil palm-derived EFBs and incubated for six days. In the MZAC, the fungal microbes were left freely suspended in the electrolyte. They eventually grew and attached themselves to the EFBs at the bottom of the cell enclosure. Refer to our previous publication for details [8]. The electrolyte was not buffered; no additives, such as an electron transfer mediator, microbe growth enhancer, or particular nutrients, were added. The MZAC was left to operate in an uncontrolled environment.

2.3 Operating principles of the microbial zinc/air cell (MZAC)

When supplied with lignin-rich EFBs, *P. chrysosporium* fungal microbes will secrete ligninolytic enzymes that predominantly contain laccase, which has a specific affinity for O_2 molecules as its electron acceptor. Therefore, a bio-catalyzed electrochemical MZAC can be produced by pairing a zinc anode with an air cathode in a medium rich with *P. chrysosporium* hyphae. While the fungal microbes degrade the lignocellulosic wall, the laccase will catalyze the reduction of molecular O_2 [11].

2.4 Influence of electric current stimulation (ECS) on the lignin degradation (LD) of empty fruit bunches (EFBs)

The influence of ECS on the LD of EFBs was observed under two conditions:

(a) Discharge current from MZAC
ECS was generated from the discharge current of the MZAC. A discharge current of 1, 2, and 3 mA was produced continuously for 30 days.

(b) External current
The same MZAC enclosure was utilized, but both electrodes were replaced by nickel mesh. A constant external current of 1, 2, 3, 5, and 6 mA was applied continuously for 30 days.

The current was regulated using a NEWARE® BTS4000 battery tester for both conditions. After 30 days of discharge, the EFBs were retrieved, rinsed, and dried for characterization.

2.5 Characterising the lignin degradation (LD) of the empty fruit bunches (EFBs)

The LD of the EFBs was characterized according to their surface morphology and quantitative lignin content (LC). Changes to the surface morphology were observed using a JEOL® JSM-6700F scanning electron microscope (SEM). The quantitative LC was determined using the acid-chlorite method. In this method, 0.15 g of EFBs was first boiled twice with 75 ml of H_2O for 1 hour to remove the hot-water-soluble materials before being dried at 60 ℃ for 15 hours and weighed (W_1) [12]. The samples were treated with 30 ml of H_2O, 2 ml of 10% acetic acid, and 0.6 g of sodium chlorite at 75 ℃ for 1 hour. Then, they were treated further with 10% acetic acid (2 ml) and 0.6 g of sodium chlorite at 75 ℃ for 2 hours. Finally, the samples were rinsed with H_2O (five times), acetone (twice), and ether (once) and then dried at 105 ℃ for 90 minutes and weighed (W_2). All samples were tested in triplicate. The LC was determined using:

$$Lignin\ content\ (\%) = \frac{W_1 - W_2}{0.15}, \quad (1)$$

3. Results and Discussion

Empty fruit bunches (EFBs) are a lignocellulose waste of lignin, cellulose, and hemicellulose. Lignin is the most chemically resistant to decomposition among the three components due to its complex structure. It is formed by the oxidative coupling of three distinct phenylpropane building blocks: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, connected by carbon-carbon and ether bonds [13]. Due to the heterogeneity of the bonds, instead of hydrolytic enzymes, ligninolytic enzymes are required for delignification, and basidiomycetes WRF has been proven to be the most effective.

The basidiomycetes WRF, *P. chrysosporium*, is highly capable of degrading lignocellulosic biomass by secreting enzymes such as laccase, MnP, LiP, xylenes, cellulase, glucose-1-oxidase, and glucose-2-oxidase [14]. Compared to other WRFs, *P. chrysosporium* possesses the advantages of a high growth rate, adaptability under harsh environments, simultaneous decay of lignocellulosic materials, and excellent oxidation abilities [13,15].

It was observed that upon exposing the EFBs to *P. chrysosporium*, the outer protective lignin layer showed signs of degradation starting on Day 20. By Day 30, the cell walls had almost wholly collapsed [8]. The efficacy of the LD of the EFBs under ECS was also characterized based on the estimation of the LC after 30 days of exposure to *P. chrysosporium*. Changes to the morphology of the EFB substrate were also observed to support the quantitative data. The chemical structure of lignin and its abundance, even from the same plant, vary with its source. These characteristics contribute to the lignin-breaking behavior as well [16]. However, the structural characteristics of lignin are often overlooked when discussing LD.

As anticipated, the ECS strongly influenced the LD of the EFBs using *P. chrysosporium*. The lignin composition in EFB was estimated at around 34.0%. In the absence of the ECS, after 30 days of exposure to *P. chrysosporium*, the EFBs' LC was reduced from 34.0% to 28.4%. At a discharge current of 1 mA, the LC was 24.2% (~14.8% decrease); at a discharge current of 2 mA, the LC was 20.3% (~28.5% decrease); and at a discharge current of 3 mA, the LC was the lowest, i.e. 17.9% (~37% decrease) (Table 1). Therefore, the discharge current promoted the LD of the EFBs using *P. chrysosporium,* and the influence increased as the DC increased up to 3 mA. The cell could not sustain a constant discharge current greater than 3 mA.

Table 1. The LC is a function of the ECS (MZAC's discharge current).

LC (%)						
Control	**Post-30 Days Exposure to a Constant DC**					
No Current	1 mA	Δ (%)	2 mA	Δ (%)	3 mA	Δ (%)
28.4 ± 0.2	24.2 ± 0.2	14.8	20.3 ± 0.02	28.5	17.9 ± 1.0	37.0

Δ - Decrement in LC
Each value is a mean of three replicates ± standard errors.

The presumption that the availability of free electrons promotes LD by *P. chrysosporium* was tested by applying an external current to an enclosure containing the EFBs and *P. chrysosporium*. Both the zinc anode and air cathode were replaced with nickel mesh. An electric current stimulation (ECS) was externally supplied to pass through the enclosure. The percentage of remaining LC as a function of the external current is presented in Table 2. The results substantiated the role of an external current in promoting LD using *P. chrysosporium*. Interestingly, the external current had a more substantial influence on LD as the LC of the EFBs was lower than that of the MZAC discharge current. It was thought that in the MZAC, a portion of the discharge current participated in the electrochemical redox reactions, while the remaining was utilized in the fungal metabolic activities. On the other hand, when an external current was applied in the absence of the anode-cathode couple, the entire external current could be utilized in the fungal metabolic activities, thus contributing towards a higher LD. When the external current was 3 mA, the fungal LD rate was the highest and even doubled under its influence. However, the fungal LD rate did not increase when the external current increased. When the external current was 5 and 6 mA, the LC was roughly similar to when the external current was 1 mA. Figure 1 summarises the variations in the LC of the EFBs as a function of the ECS.

Table 2. The LC is a function of the ECS (external current).

LC (%)										
Control	**Post-30 Days Exposure to a Constant DC**									
No Current	1 mA	Δ (%)	2 mA	Δ (%)	3 mA	Δ (%)	5 mA	Δ (%)	6 mA	Δ (%)
28.4 ± 0.2	22.8 ± 0.1	19.7	17.1 ± 0.8	39.8	7.5 ± 0.01	74.6	22.1 ± 0.5	22.2	22.2 ± 4.0	21.8

Δ - Decrement in LC
Each value is a mean of three replicates ± standard errors.

Beyond 3 mA, the supply of free electrons no longer increased the LD. There are two possible explanations. First, the excess charge supplied might have disrupted or inhibited the fungal metabolic activities, and hence, they were no longer at an optimum rate [4]. Second, with an increasing supply of free electrons, more reactive oxygen species would have been generated, which, in turn, would have produced more phenoxyl-free radicals. However, when too many phenoxyl-free radicals are generated, they can react with other organic compounds, such as cellulose and hemicellulose. This probably contributed to the high standard error in determining the LC at 6 mA. Brenelli et al. [17] found that in a laccase-mediated system, electrons from the delignification of lignin can be used to activate lytic polysaccharide monooxygenases and, ultimately, enhance the enzymatic hydrolysis of cellulose.

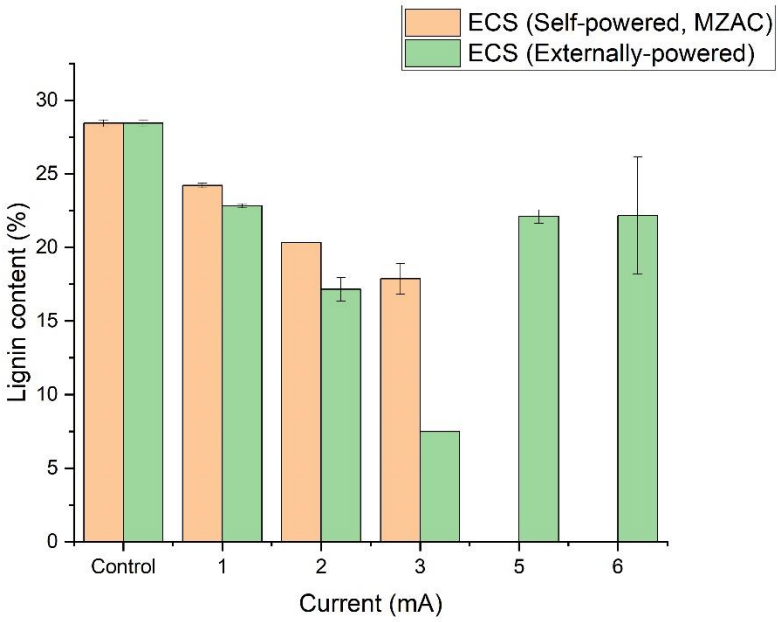
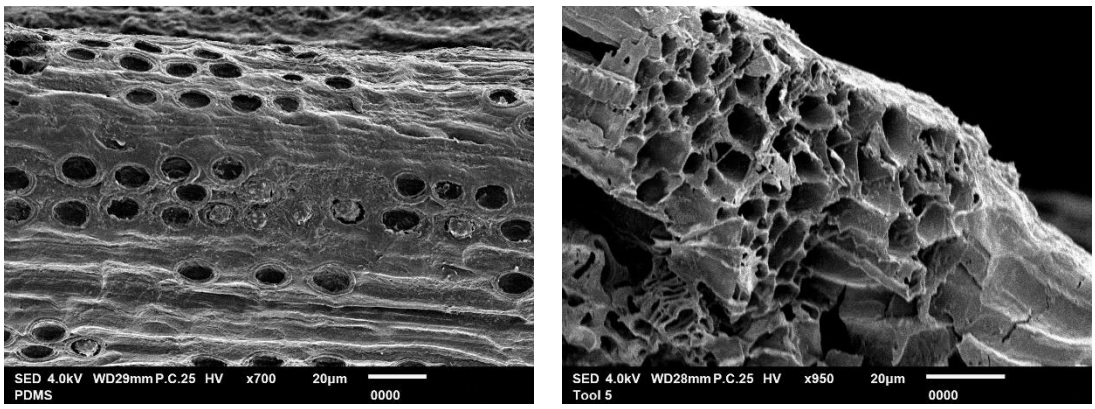


Figure 1. The influence of ECS on the LD of the EFBs using *P. chrysosporium*. Each value is a mean of three replicates ± standard errors.

The LD of EFBs by *P. chrysosporium* under ECS was also characterized by observing the changes to their morphology. Figure 2 shows the scanning electron microscope (SEM) images of an EFB strand. It had a rigid and rough surface with craters and silica bodies attached (Figure 2a), preventing fungal hyphae from penetrating the cellulose and hemicellulose matrix. Once the silica bodies are removed from the surface, it will be easier for fungal hyphae to penetrate through for delignification. Figure 2b suggests that each EFB strand comprised rod-shaped microfibrils lumped with cavities in the middle. Each rod-shaped microfibre was surrounded by cell walls containing most of the lignin [3,18].

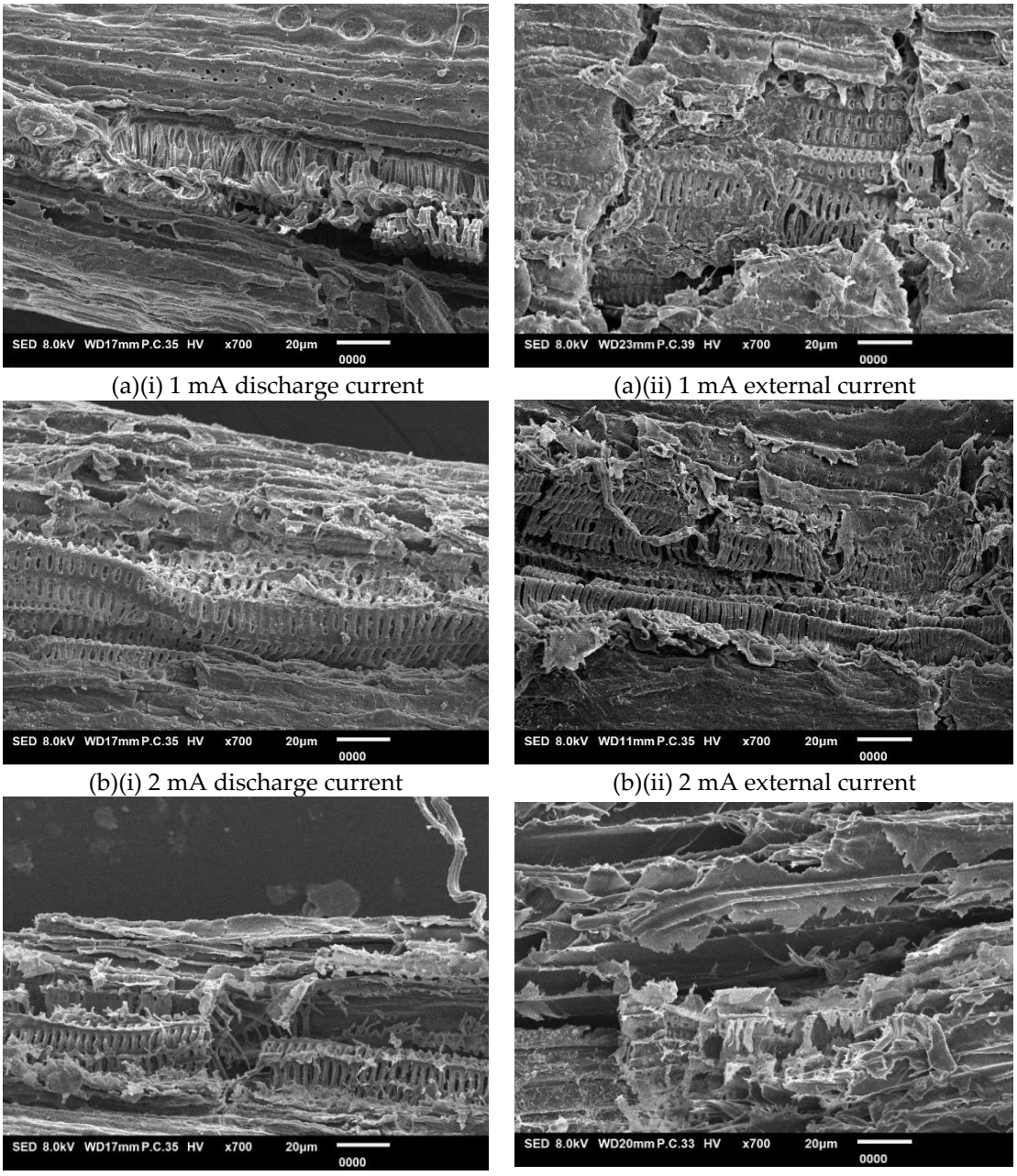


(a) Longitudinal view (b) Cross-sectional view

Figure 2. SEM images of the morphology of the EFB strand pre-exposure to P. chrysosporium.

Figure 3 shows the morphology of the LD of the EFBs using *P. chrysosporium* and after 30 days of exposure to varying ECS. The observations clearly showed that the ECS enhanced the LD of the EFBs using *P. chrysosporium*, as substantiated earlier by the determination of the LC. At ECS = 1 mA (Figure 3a), the outermost cell wall had almost wholly degraded, showing a pitted vessel and helical/matrix-like structure attributed to cellulose [19]. More pitted vessels were visible at ECS = 2 mA, exposing a more matrix-like structure and allowing the *P. chrysosporium* mycelium to penetrate the innermost cell wall (Figure 3b). Finally, at ECS = 3 mA, most of the outermost cell wall and the outermost matrix-like structure had almost completely

collapsed, revealing the inner vessels and matrix-like structure. From the SEM images, the external current yielded more a significant LD in the EFBs, as proven by the lower LC.



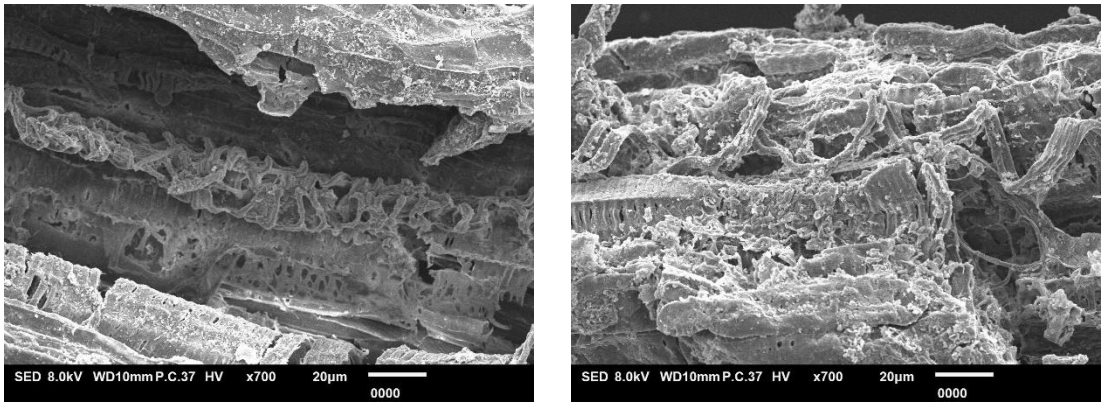
(a)(i) 1 mA discharge current (a)(ii) 1 mA external current

(b)(i) 2 mA discharge current (b)(ii) 2 mA external current

(c)(i) 3 mA discharge current (c)(ii) 3 mA external current

Figure 3. SEM images of an EFB strand morphology post-30 days of exposure to ECS: (i) MZAC discharge current and (ii) external current.

Figure 4 shows the SEM micrographs of the LD of the EFBs using *P. chrysosporium* when the external current was 5 and 6 mA. At first glance, the LD appeared to be advanced. However, this was not supported by the acid-chlorite LC data. Upon careful observation, the inner vessels and matrix-like structure, which corresponded to the cellulose and hemicellulose, collapsed faster than the protective outermost structure, i.e., the lignin layer. This was, most likely, due to a reaction of the excess phenoxyl-free radicals.



(a) 5mA external current (b) 6mA external current

Figure 4. SEM images of the LD of EFBs using *P. chrysosporium* and external current of (a) 5 mA and (b) 6 mA.

Shen et al. [4] reported the enhancement of LD by *T. versicolor* upon inducing an electrocatalytic electro-Fenton reaction for 96 hours. The study utilized a commercial de-alkaline lignin. They reported an increase in LD from 25 to 85.4% when the current was increased from 50 to 300 mA. The electro-Fenton method is an electrocatalytic reaction based on the in-situ generation of hydroxyl radicals on an iron-based composite cathode (Fe^0 and Fe_3O_4/graphite felt). A huge amount of hydroxyl radicals are produced from the reaction between hydrogen peroxide (H_2O_2) and Fe^{2+} ions. The strong hydroxyl oxidant is primarily thought to degrade the complex lignin structures and functional groups. Consequently, this will promote the acceleration of LD by LiP and MnP.

Beschkov et al. [10] and Vasileva et al. [20] studied the effect of a constant EC on phenol degradation using the bacteria *Pseudomonas putida* and *B. japonicum 273*. The enhanced phenol degradation (3 - 4 times) was attributed to the ECS generated by the activities of the excreted enzymes (phenol hydroxylase and catechol dioxygenase) rather than the electrochemical oxidation at the anode. They applied an anodic potential of 0.8 - 1.0 V (SHE) to a fed-batch mode fermenter with an initial concentration of 0.06 g/L of phenol. They reported that the highest phenol removal efficiency was observed at 0.8 V using *P. putida* and 1.0 V using *B. japonicum 273*. For most of the experiment, the discharge current was < 10 µA [10]. They conjectured that the applied external current induced some changes at the site of the active enzymes and stimulated the activation energies of phenol oxidation and benzene ring cleavage [10].

The present study examined the LD of EFBs using *P. chrysosporium* and a constant ECS. *P. chrysosporium* secretes ligninolytic enzymes such as laccase and H_2O_2-dependent ligninase (i.e., LiP and MnP). All these enzymes are thought to act synergistically to degrade lignin and other biopolymers [21]. Given the aerobic nature of the fungal degradation of wood, the enzymatic processes are directly or indirectly (via intermediatory substrates) oxidative [16]. There is no general agreement on the enzymatic LD mechanisms due to two key variables: the heterogeneous lignin source and unpredictable lignin structure [16], let alone the type of fungal species involved. A typical laccase-based LD is commonly described as follows: Laccase catalyzes the one-electron oxidation of lignin phenolic units into phenoxyl-free radicals and electrons. These free electrons are then transferred to the side chain, leading to the cleavage of bonds (C_α-C_β cleavage, alkyl-aryl cleavage, and C_α oxidation) [22]. Manganese peroxidase (MnP) and LiP, on the other hand, act by producing mediators of oxidative LD (i.e., chelated Mn^{3+} and veratryl alcohol radical cations) [16,23]. Laccase, in turn, also possesses the ability to act as a mediator of the reactive products formed above [16].

Regardless of the proposed LD pathways using fungal microbes, they are characterized largely by electron transfers and radical formations [24]. One of the roles of the produced radicals is to serve as electron acceptors/mediators [24,25]. Thus, the present study posits that if free electrons are abundant during the LD of fungal microbes, this can lead to the cleavage of more bonds and eventually promote LD. Enhancing the rate of LD is a key step in the economic and green decomposition of lignocellulosic agricultural waste. This study was able to successfully develop an LD method that is much cheaper, significantly less complicated, and requires significantly less current than the electro-Fenton method.

4. Conclusions

The present study has substantiated that LD can be enhanced by nearly 74.6% by merely supplying free electrons to *P. chrysosporium*-cultivated EFBs. The electron supply can be generated in situ by the insertion of redox couple electrodes. This approach is much cheaper than the electrocatalytic reactions of the electro-Fenton method.

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