Investigating fungal laccase diversity for putative plastic degradation activity

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Reviewed on 8 May 2021; Accepted on 28 June 2021; Published on 25 October 2021

Petroleum-based plastic is an environmental concern due to the difficulty of its degradation, leading to the persistence of plastic waste. As research into this material continues, it is imperative that solutions to curb this accumulation of waste, including scientific ones, be prioritised. Laccases are a group of carbohydrate-degrading enzymes found across different organisms with putative activity related to plastic degradation and thus is a protein of interest. This paper aims to investigate potential methods to degrade single-use plastic, focusing on different laccases in fungi, such as that of *Pleurotus ostreatus* and explores laccase diversity through homology searches to determine the presence of these enzymes in different fungal species and their evolutionary relationship. Eventually, we plan to study the functional expression of these laccases secreted from *Pleurotus ostreatus* grown on plastic using quantitative polymerase chain reaction (qPCR) and protein gel electrophoresis. Understanding the diversity of laccase genes and their roles in plastic degradation will allow development of synthetic biology solutions, including plastic degradation devices using laccases. This critical first step is needed to enable better standardization of parts to build systems for plastic degradation that can be used in other microbial or eukaryotic chassis.

Keywords: *Laccase diversity, Pleurotus, plastic degradation, phylogenetic tree*

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Watch a video introduction by the authors at https://youtu.be/DQyeAHenqL8

Background

A great environmental concern is plastic pollution and persistence. Plastics are made of monomers distilled from petroleum that join together when heated in the presence of a catalyst to form polymers, for instance propene, a monomer, into polypropene (polymer). The largest issue is that they generally do not biodegrade because when these monomers join together, they form strong non-polar carbon-carbon bonds. Since plastic is manmade and non-reactive, the natural world has not encountered such carbon-carbon bonds, so there is a lack of metabolic pathways dedicated to break them (Wolchover, 2011). As polar bonds found in nature (such as peptide bonds) are more reactive and take less energy to establish (in comparison to carbon-carbon bonds), they can be broken apart by natural enzymes. Thus, this difficulty in biodegrading plastic results in its accumulation. However, as interest in this issue of plastic persistence grows in prominence,

methods have been and are being studied to curb the aforementioned issue.

The realm of fungi is still a largely unexplored field in terms of its potential mechanisms regarding what its members may be able to break down, including plastic. However, in recent years there has been an increased interest in fungal contribution to plastic degradation. *Pleurotus ostreatus*, the common oyster mushroom, has particularly been of interest as research by da Luz et al. (da Luz et al., 2013). has shown its ability to degrade plastic. There are multiple enzymes in *P. ostreatus* which may have a role in the degradation of plastic, mostly lignocellulolytic enzymes including manganese peroxide, xylanase, cellulase, and laccase (da Luz et al., 2013; Sivan, A. 2011). While, the degradation of plastic was largely due to laccase activity. Laccases are enzymes that fall under the phylum of multicopper oxidases (MCOs) (Janusz et al., 2020). Laccases are copper-containing proteins belonging to MCOs and able to reduce oxygen into the water; specifically with the mechanism that allows the transfer of hydrogen atoms to an oxygen molecule to produce hydrogen peroxide or water (Yang et al., 2017).

Aside from this ability and their putative activity in degrading plastics, laccases were chosen because of their divergence, meaning there are several genes that code for laccases even in a single organism. Previous investigations,on the laccase gene family of cotton, demonstrated the presence of up to 84 different laccase proteins. Two other varieties of cotton were found to have 40 and 44 different laccase proteins (Balasubramanian et al.2016). We hypothesize that there are multiple laccase genes present in *P. ostreatus* that allow them to break down plastic. Furthermore, high diversity in laccases of *P. ostreatus* may point to a higher chance that one of the laccases has a role in single-use plastic degradation. We intend to group the laccases by the degree of homology to identify unique enzymes to *P. ostreatus* that may have a functional role in degrading plastic.

Materials and Methods

The nucleotide sequences of whole and partial laccases were identified using the online database CAZy (www.cazy.org), and nucleotide sequences were retrieved NCBI GenBank database (www.ncbi.nlm.nih.gov/genbank). A randomly selected laccase nucleotide sequence was run into the NCBI BLAST program (www.blast.ncbi.nlm.nih.gov/Blast.cgi). After the BLAST, the first focus was on the list of laccases from the CAZy database. The accession codes for the selected laccases were checked and compared to the organisms that have shown a high percentage identity. If any of the accession codes were that of laccases in the spreadsheet, they were then grouped and labelled into one cluster, numbered for ease. The percentage similarity between the sequences was recorded. Furthermore, after the BLAST program had been run, all the sequences of the organisms which have sequences that produced alignments with significant sequence similarity were downloaded in a FASTA formatted file. The FASTAs were labelled according to their cluster, the first letter of the genus and species, and the accession code. An example is '>Clus4-PO-AB551114.1' with Cluster 4, binomial name *Pleurotus ostreatus*, and its accession code. The laccases which do not have a high similarity (in that they were not on the BLAST list) were individually BLASTed using the same method. The accession codes that appeared from this second BLAST were compared to the list on the spreadsheet. Those that were significantly similar to this second BLAST run were also recorded and grouped into clusters. These processes were repeated until all the laccases had been grouped into clusters through BLAST or based on the similarity levels. All the sequences that have appeared from the BLAST were recorded into the same document containing previous sequences.

Table 1. Data of different laccase clusters formed based on BLAST data

No.	Cluster	Protein name	Organism	Accession No.	Nucleotide percentage similarity (no. - %)
A	1	laccase, partial (fragment)	*Pleurotus ostreatus*	KX838288.1	H - 97.68
B	4	laccase (Lacc6)	*Pleurotus ostreatus HAUCC 162*	KX815352.1	-
C	2	laccase	*Pleurotus ostreatus*	KX782335.1	-
D	3	laccase (short fragment)	*Pleurotus ostreatus*	JN367282.1	-
E	5	laccase (Lacc9)	*Pleurotus ostreatus HAUCC 162*	KX815353.1	E - 100
F		laccase, partial (fragment)	*Pleurotus ostreatus U1/9*	KT447641.1	E - 96.08
G		laccase	*Pleurotus ostreatus*	AY485827.1	E - 99.50
H		laccase	*Pleurotus ostreatus*	AY450404.1	E - 84.29
I		laccase, partial	*Pleurotus ostreatus*	MT313303.1	E - 84.14
J		laccase	*Pleurotus ostreatus*	KC789847.1	E - 91.32%
K		laccase, partial (fragment)	*Pleurotus ostreatus A12*	MG717888.1	E - 84.99
L		laccase (Lacc10)	*Pleurotus ostreatus HAUCC 162*	KX815354.1	E - 84.02
M		laccase / diphenol oxidase (Pox2)	*Pleurotus ostreatus NRRL0366*	Z49075.2	E - 85.89
N		laccase, partial (fragment)	*Pleurotus ostreatus U9/2*	KT447636.1	E - 95.42
O	15	laccase, partial (fragment)	*Pleurotus ostreatus U11/2*	KT447637.1	F - 100

The (FASTA) sequences that had been labelled accordingly were copied onto Clustal Omega for data processing. A phylogenetic tree was generated by changing the tab options to phylogenetic tree to compare how closely

related the sequences of one cluster are. This also allowed the analysis of whether the sequences of laccases were identical, and whether all laccases were coded by the same gene, and showed which sequences shared higher percentage similarities, or which clusters were closely related.

Results

Data of different laccase clusters formed based on BLAST data

A FASTA file containing all sequences that were BLASTed according to the data in Table 1 was made. The file was used to create the phylogenetic tree found in Figure 1 and Figure 2.

Phylogenetic tree showing specific monophyletic groups

The phylogenetic tree indicates that there are distinct monophyletic groups for the laccases of *P. ostreatus*, as it is for some other species, such as for *Pleurotus* eryngii. Monophyletic groups are the descendant species all related to a single ancestral species, which have evolutionarily branched out and diverged from the ancestral sequence.

Figure 1 shows a phylogenetic tree for the laccase sequences that were collected from the BLAST search, showing how similar each gene is as real distances are used to scale. It should be noted that there were some errors in recording data from the BLAST, so there are some duplicates of the same laccase, and some are categorized in different clusters. However, the accession codes on the tree are visibly seen and duplicates are identified. There are two distinct monophyletic groups of *P. ostreatus*, labelled with PO, highlighted with red rectangles. Furthermore, there is another group for *P. ostreatus* (shown with a blue box) clustered with other species. These branching patterns, which show the differences in monophyletic groups that exist, support the idea that there may be more than one copy of laccase. It is also further supported by data found in the CAZy database, which shows that the same strain of *P. ostreatus* has multiple laccases with different accession codes. For example, the subspecies *Pleurotus ostreatus* HAUCC 162 has three different laccases, Lacc6 (KX815352.1), Lacc9 (KX815353.1), and Lacc10 (KX815354.1). Thus, there are multiple copies of laccases in different strains of said species. Since multiple copies are found, this may lead to the idea that there is variation in laccase specificity and activity, wherein one may have potentially developed the ability to degrade plastic.

A possible explanation for the two monophyletic groups in Figure 1 (in the red rectangles) is that they were evolved by an evolution. Findings from another study support

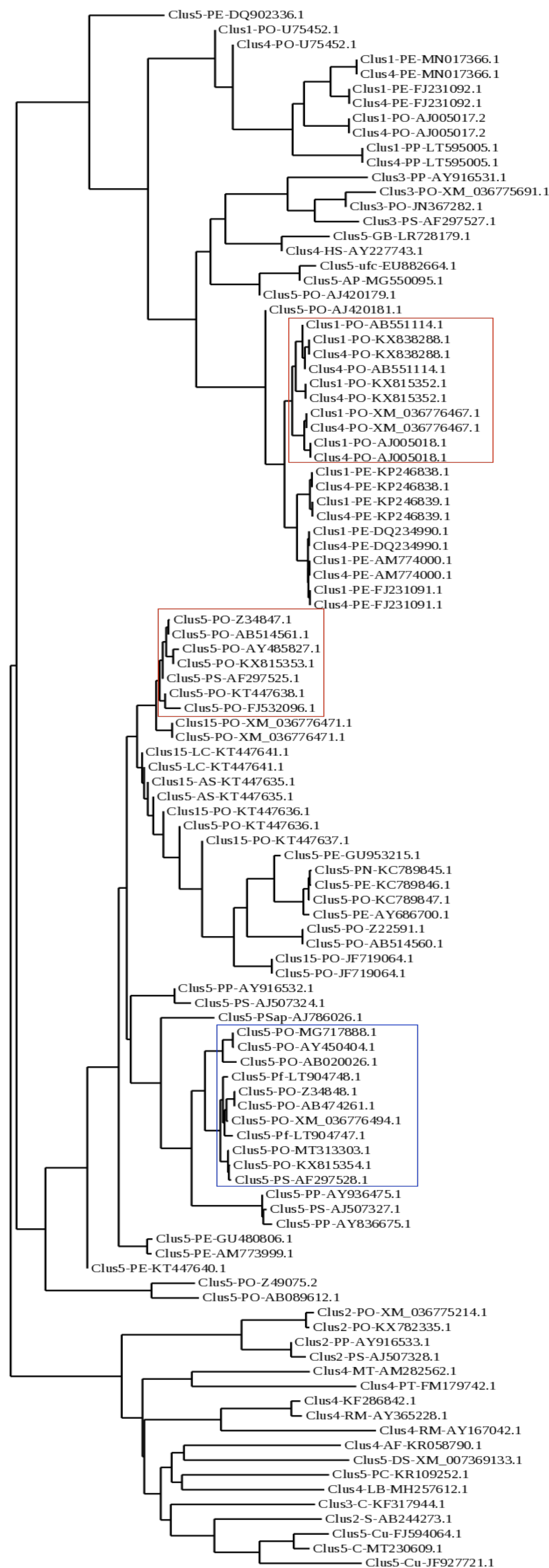

Figure 1. *Phylogentic tree of laccases nucleotides*

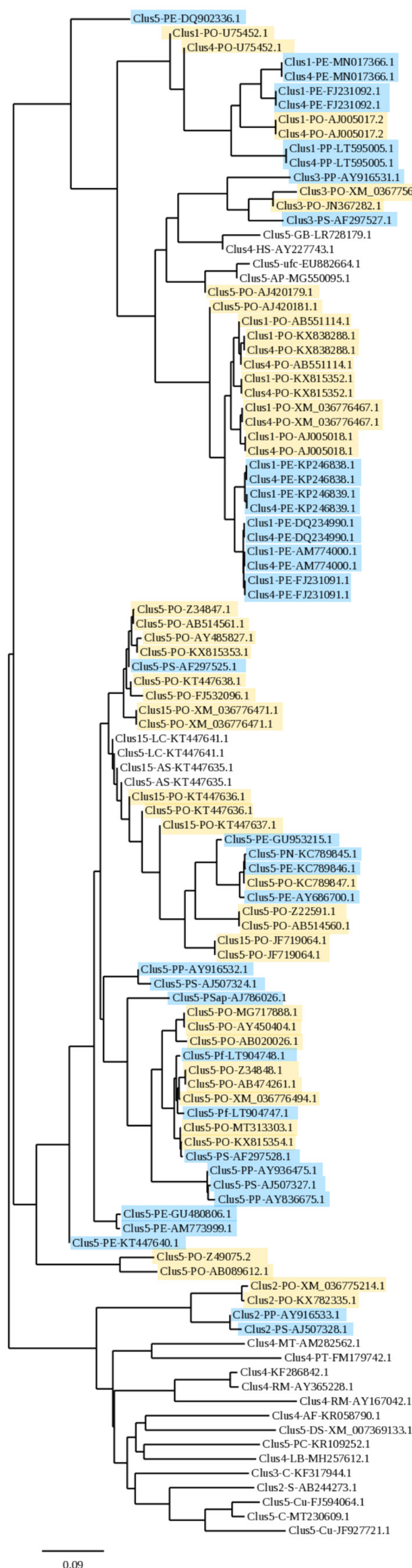

Figure 2. *Phylogentic tree of laccases. Pleurotus is highlighted in yellow. Remaining Pleurotus genus is highlighted in blue*

the idea of evolution leading to more laccase varieties, wherein they stated that in cotton plants, segmental and tandem duplication events as a part of the cotton evolution process led to an increased number of laccase genes (Balasubramanian et al., 2016). Furthermore, sequences in different monophyletic groups with other organisms require further investigation to identify their ability. From another stance, it may not be too extreme to state that fungi and laccase proteins' evolution may be driven by changes in environmental aspects and food sources, such as plastic. Thus, we hypothesize that this diversity may allude to the idea that laccases can break down plastic at a molecular level. Currently, we are looking at specific mutations and mapping them onto laccase structures to determine whether the hypothesis is supported.

The possibility of the fungi in the Pleurotus genus breaking down single-use plastics

There is still much to discover and learn about the field of fungi and its mechanisms and functions (Li, 2020). However, da Luz et al. (2013) showed that laccase, among other enzymes, did show increased activity when grown on plastic, suggesting the involvement of the enzyme in the degradation of plastic. Given that there is still much that we need to explore about fungi, it is reasonable to hypothesize that the plastic-degrading ability currently accredited to *P. ostreatus* might be present in other Pleurotus species. This concept is supported by Figure 2. Figure 2 shows the phylogenetic tree of laccases from *P. ostreatus* highlighted in yellow and other *Pleurotus* fungi in blue. This homology tree shows that the laccases of different species of *Pleurotus* are similar to that of *P. ostreatus*, for example, *Pleurotus eryngii* - signified by PE - which forms its own monophyletic groups, such as below the top-most *P. ostreatus* monophyletic group in Figure 1. This relatively high similarity between the laccases, as shown by short lines on the phylogenetic tree, among different *Pleurotus* species leads to the hypothesis that laccases of other fungal species under said genus also have the ability to degrade plastics. Even so, it must be considered that the genetic divergence may lead to lack of plastic degradation function in other *Pleurotus* species. As such, more studies should be carried out to investigate this in the future, as will be detailed in the following sections.

Discussions

The proposed project was - first and foremost - free. It involves utilizing existing databases as a foundation for understanding laccases, and this information may later be applied to wet labs and physical means of testing. The use of pre-existing databases means these procedures can be easily carried out by anyone and this accessibility allows for even more further development. The accessibility of

this method of experiment means that it can be repeated, and the information collected can be supported or even disputed.

Having a wide array of information available also allows the study of different laccases of different organisms, which enriches public knowledge and will enable comparisons related to monophyletic groups, amino acid sequences, and much more. In a wet lab, it is likely that an experiment requires a certain focus and setup that require a set amount of materials. This could mean that expansion of such experiments would require more preparation and expenses. However, a database lab allows for more flexibility and virtually no incurred cost if more is explored.

However, there are a number of challenges that this project presents. Although using databases allows for a wide array of analysis, there has been no concrete method used to determine whether laccase works independently or in coordination with another mechanism. This unknown may be especially significant as the plastic degrading ability of laccase is still putative. Furthermore, the use of a database means that this experiment is solely based on secondary data, and so although the results generated from this are supported, it is an ongoing hypothesis.

Another challenge that comes about is that there is still much more to learn about laccases and fungi. Since secondary data were utilized, the information in databases about laccases and fungi is quite limited, especially since one database only focuses on a narrow aspect, such as nucleotide and protein sequences.

Next Steps

Since this project was reliant on database sources, a practical experiment in the laboratory can be carried out with *P. ostreatus*. This would include growing the mycelia of *P. ostreatus* on the plastic substrate and can be compared with control (*P. ostreatus* on a regular growth substrate). This method will allow investigating whether or not the mycelium has broken down the plastic (tensile strength of the plastic can be demonstrated by microscope) and to compare the activity of *P. ostreatus*. An enzymatic assay can be used to assess the difference in laccase activity between fungi treated with plastic and without. As another extension for more analyses related to this growth of fungi, different *Pleurotus* fungi can also be grown and analyzed to see whether they have the ability to degrade plastic. If they do not, there can be a comparison between which parts of the laccase sequence are different between *P. ostreatus* and other fungi.

More complete data can potentially be collected after the growth of the mycelia using qPCR and RNA sequencing. This is useful in identifying the differences between the genes expressed when *P. ostreatus* is grown on plastic compared to without. The process would provide the specific differences between the genes expressed for further analyses. Eventually, certain genes or proteins may be isolated to investigate their functions in degrading plastics, and from here designs may be developed to cultivate them, making the breakdown of plastic more effective. However, since there may be issues with the accessibility of RNA sequencing, alternatives such as protein gel electrophoresis may be used instead, although less specific. If specific proteins of the fungi have been identified from the database analysis and targeted, qPCR may be used instead of RNA sequencing to observe the changes in gene expression.

Using the data analyzed in the results, the nucleotide sequences can be translated into proteins to further pinpoint how the laccases of *P. ostreatus* or *Pleurotus* groups differ from other laccases. Differences or unique proteins may support the hypothesis of such fungi having a functional role in degrading plastic. Amino acid sequences can be compared to exemplar or well-analyzed laccases of other fungi, using databases such as the RCSB Protein Data Bank.

Alignments have shown that there are definite differences between the sequences of laccases within the same species, but these differences may be due to introns or silent mutations that may not affect the function of the proteins. The well-studied laccase would be useful when aligning it with the amino acid sequence of *Pleurotus* laccases as the positions of the amino acids can be checked to see whether or not the essential parts are different from each other. For instance, a different sequence for the binding site of the enzyme can change a pivotal role that the ancestral laccase had, and therefore change the function of the laccase itself. The high percentage of diversity between alignments of amino acids sequences for essential parts would likely further support *Pleurotus*' putative ability to degrade plastic.

Author Contributions

CJH helped heavily with the research and tools used to analyze the data collected from the CAZy database, with great help in processing the information. Research on the background of the situation was conducted and the processed information was converted into written form for the manuscript by RW. Raw data in regards to sequences and data for different laccases and their accession codes were collected by RW. JB recommended the tools for the methodology and partook in the writing of the manuscript. The methodology and materials of this project were developed by JB, CJH, and RW collectively, in addition to the analysis of the collected data and results derived from raw data.

Acknowledgements

This manuscript could not have been complete without the tireless efforts of Christopher Joseph Hayden and Dr. Jason Boock, who provided extensive information on the background to understand the concepts and tools explored in this manuscript. They have also dedicated an immense amount of time to this paper and project. The development of this project could not have reached this extent without the generous grant funding from the IB MYP Innovator's Program held by the International Baccalaureate. All authors acknowledge with gratitude the commitment of the funder to its research efforts.

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