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Review

# Fungal Assisted Valorisation of Polymeric Lignin: Mechanism, Enzymes and Perspectives

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**Abstract:** Lignocellulose is considered one of the significant recalcitrant materials and also is difficult to break down because of its complex structure. Different microbes such as bacteria and fungi are responsible for breaking down these complex lignin structures. This article discussed briefly the lignin-degrading bacteria and their critical steps involved in lignin depolymerization. In addition, fungi are regarded as the ideal microorganism for the degradation of lignin because of their highly effective hydrolytic and oxidative enzyme systems for the breakdown of lignocellulosic materials. The white rot fungi, mainly belonging to basidiomycetes, is the main degrader of lignin among various microorganisms. This could be achieved because of the presence of lignolytic enzymes such as laccases, lignin peroxidases, and manganese peroxidases. The significance of the fungi and lignolytic enzyme's role in lignin depolymerization, along with its mechanism and chemical pathways, are emphasized in this article.

**Keywords:** depolymerization; bacteria; fungi; lignin; lignolytic enzymes; pathways



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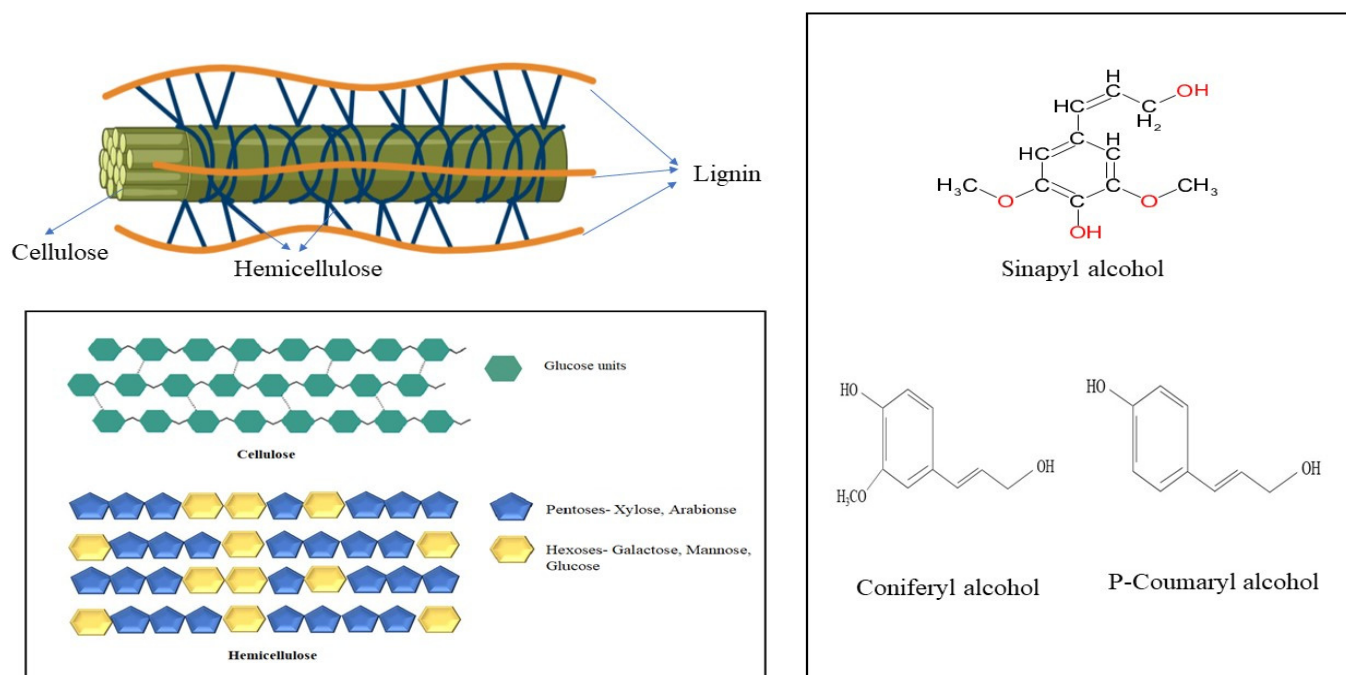


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## 1. Introduction

Agricultural leftovers, crops, yard trimmings, and forestry residues are a few examples of lignocellulosic biomass, which are plants or plant-based substances that are not utilized for food or feed and are largely available throughout the year [1]. They are essential components of plant woody cell walls and have a complex structure and are mostly proteins, polysaccharides, and phenolic polymers [2]. As an outcome, the surge in enthusiasm for producing biomass-derived lignocellulosic products such as chemicals, bio jet fuel, bioenergy, biofuels, and biochemicals [3,4].

Figure 1 depicts the non-uniform three-dimensional structure of lignin, cellulose, and hemicellulose, the primary components of biomass. Cellulose is an unbranched and linear polysaccharide that consists of hundreds to thousands of analogous chains of glucose units linked jointly by  $\beta$ -1,4-glycosidic bonds. The crystalline and amorphous portions of cellulose are interwoven to create the cellulose structure [5].



**Figure 1.** Components of lignocellulosic materials.

Hemicellulose is considered the most prevalent biopolymer and its structure are amorphous and random. It is made with short and high-branched heteropolymers comprising mostly of xylose, along with glucose, galactose, mannose, uronic acids, and arabinose. Hemicelluloses are categorized as xylans, xyloglucans, galactans, or mannans, depending on the kind of substituent. One of the most important components of lignocellulosic biomass is lignin, which is entwined with the linkage of cellulose-hemicellulose, making it more resistant towards physical and biological perturbations. The lignin structure is made of three distinct phenylpropane units called monolignols: sinapyl alcohol p-coumaryl alcohol, and coniferyl alcohol [5].

Fungi are the world's second-largest species in the non-animal living world. The effective degradation of lignocellulose is a constant challenge at the industrial level but in nature, microbes and particularly fungi degrade it very effectively. Many enzymes (fungal) are utilized in industrial processes such as food production, fermentation and biomass conversion to ethanol. Moreover, the genome sequencing of important fungus has the potential to reveal new perspectives on the nature and synthesis of novel physiologically active natural compounds as well as unexplored metabolic potential.

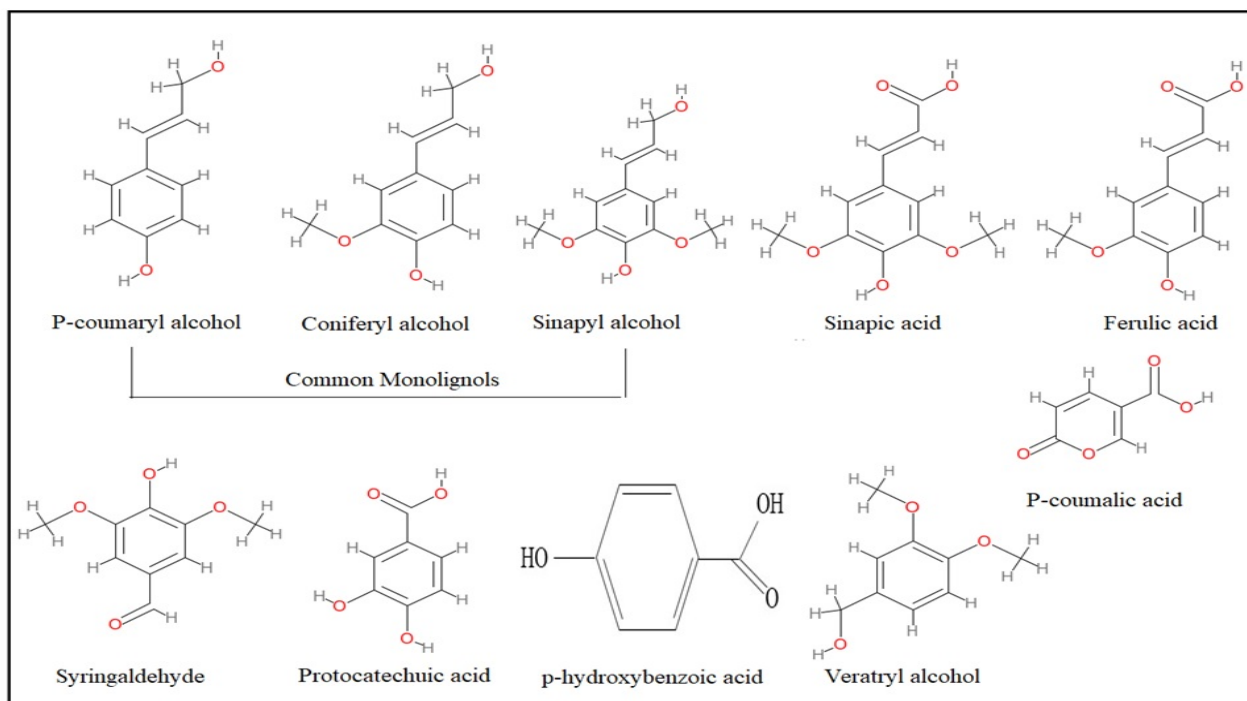
## 2. Lignin Depolymerization by Different Microbes

*Actinomycetes*,  $\alpha$ -*Proteobacteria*, and  $\gamma$ -*Proteobacteria* are the three primary classes of lignin-degrading bacteria. These bacterial species are widely distributed in the natural ecosystem and are regarded as essential decomposers of lignocellulose biomass in soils due to the formation of secondary metabolites and the utilization of extracellular enzymes. Some *Proteobacteria*, including some lineages that had not previously been involved in lignin degradation, were found to be potential polymeric lignin or aromatic fragment degraders. Some of the lignin-degrading bacteria are represented in Table 1.

**Table 1.** Different types of lignin degrading bacteria.

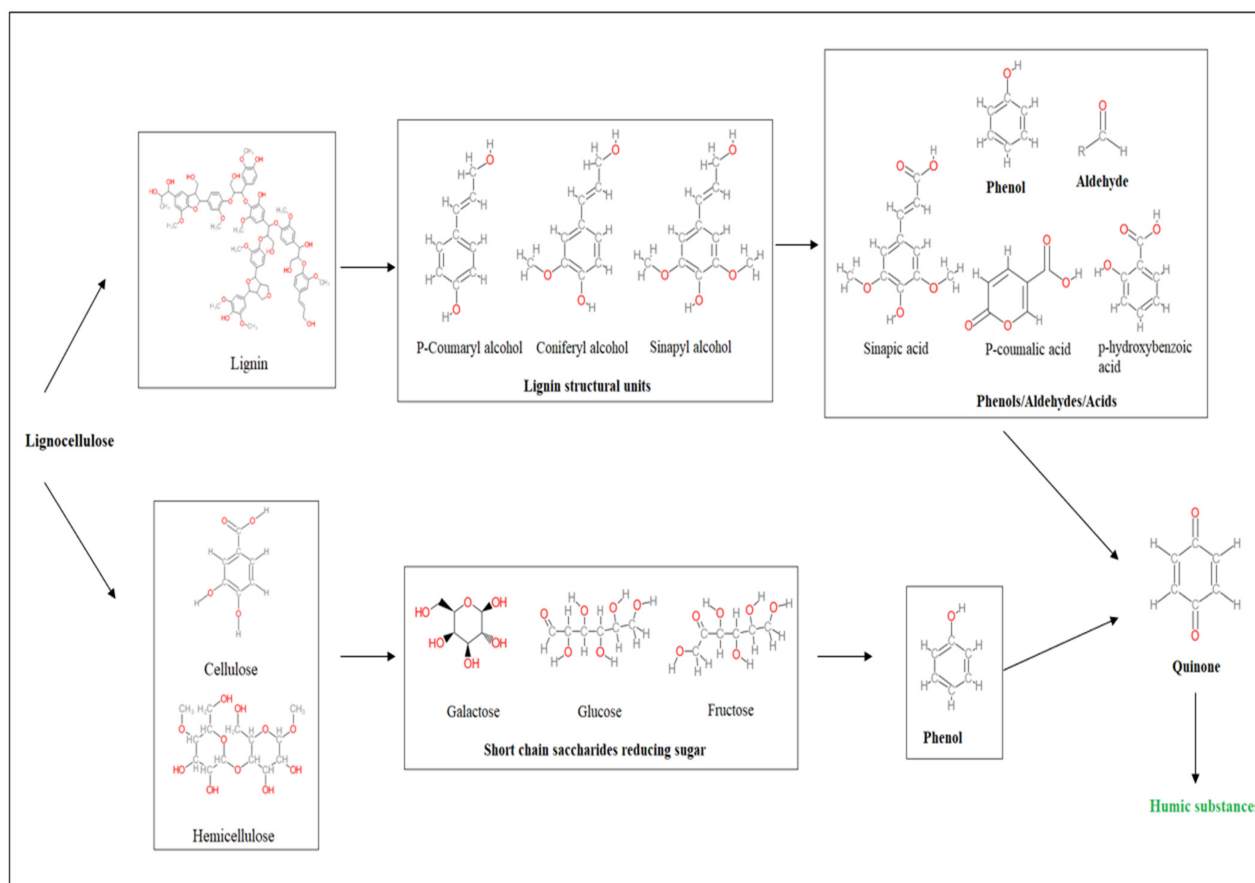
S. No	Type of Bacteria	Name of the Bacteria	References
1	Actinomycetes	<i>Streptomyces viridosporus</i> <i>paucinobilis</i> <i>Rhodococcus jostii</i>	[6–9]
2	$\alpha$ -proteobacteria	<i>Brucella</i> sp., <i>Ochrobactrum</i> sp., <i>Sphingobium</i> sp., <i>Sphingomonas</i> sp.,	[10–12]
3	$\gamma$ -proteobacteria	<i>Pseudomonas fluorescens</i> <i>putida</i> <i>Enterobacter lignolyticus</i> <i>Escherichia coli</i>	

*Proteobacteria* and *Actinobacteria* were identified as possible significant degraders of lignin-derived polymeric substances in the ecosystem. They showed that the genomes that belong to class  $\beta$ -*proteobacteria* have comprehensive enzyme methods for vanillate degradation, protocatechuate, and diarylpropanes, along with the ability to break down additional lignin-derived aromatic compounds [13]. This vanillin can be produced from lignin by depolymerization or from ferulic acid via microbial catalysis during the lignin metabolic process [14]. Some of the other lignin-derived monomeric compounds are shown in Figure 2:

**Figure 2.** Different types of lignin derived compounds.

There are mainly two steps involved in the breakdown of the lignin through microorganisms. (a) depolymerization and (b) solubilization (lignin monomers, oligomers, and their derivatives). Through these two steps, the breakdown of the lignin takes place by unlocking the inter monomeric linkages by enzymes. Figure 3. illustrates the breakdown of cellulose and hemicellulose and the formation of short-chain saccharides reducing sugars but the breakdown of lignin does not result in sugars. The lignin breakdown and solubilization can be indicated by the alterations in phenol content which are the end products of lignin

breakdown. Radhika, Sachdeva, and Kumar (2021) concluded that, for growth, microbes utilize the sugars and for the breakdown of lignin into metabolites, the obtained energy from this catabolism is potentially used [15]. It was observed that adding simple sugars as a co-substrate improves lignin depolymerization because the bacteria initiate the degrading activity on simple substrates and then move on to lignin as a secondary substrate.

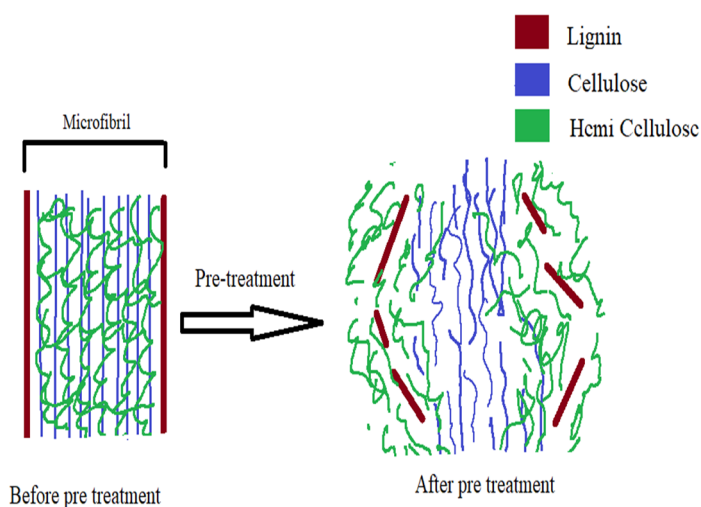


**Figure 3.** Key steps involved in lignin depolymerization in aerobic degradation.

Microbes through their arsenal of enzymes function as soil engineers and play an important role in the pre-treatment for lignocellulose degradation. The importance of biological pre-treatment of lignocellulose are represented in Figure 4 and Table 2.

**Table 2.** Advantages of biological pre-treatments [16].

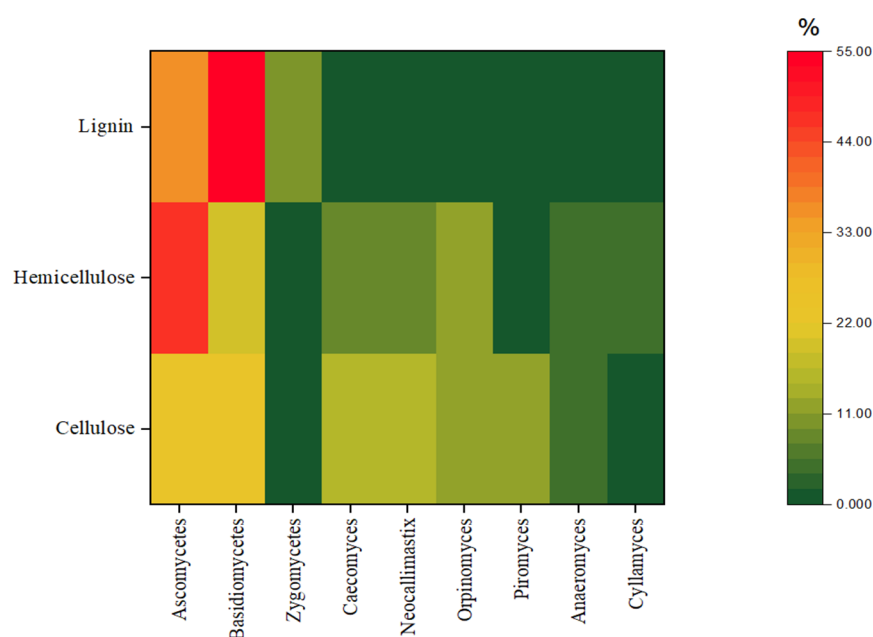
Pretreatment	Name of the Pretreatment	Advantages
Biological	Microbial	Consumption of cellulose and helicellulose No inhibitory compound formation, Low energy consumption.
	Enzymes	Alteration of cellulose structure, Delignification, Partial hydrolysis of hemicellulose, Fast process, Low energy demand



**Figure 4.** Significance of pre-treatment of lignocellulose.

### 2.1. Advantages of Fungal Microbiota

Fungi are vital in metabolizing complicated carbon sources, lignocellulosic polymer breakdown, and stabilizing organic materials in the compost system [17]. By the virtue of a highly efficient enzymatic system having hydrolytic and oxidative enzyme systems for lignocellulosic degradation, fungi are thought to be the best microorganisms for lignin breakdown. Based on their mode of degradation of lignocellulosic materials, lignin degrading fungi are diving into white rot, brown rot, and soft rot fungi. Of these white rot fungi can perform complete degradation of lignin to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ , whereas the brown rot and soft rot fungi do partial degradation [14]. *Phanerochaete chrysosporium*, a white rot fungi, is considered a model lignin-degrading organism due to its capability of degrading one gram of lignin per gram of mycelium per day. The percentage contribution of the key fungal families in lignocellulose degradation is represented in Figure 5 using a heat map plot from these studies [7,18–46]. The plot explains that mostly ascomycetes and basidiomycetes play a role in lignocellulose degradation. The list of lignin degrading fungi is given below in Table 3 [14].



**Figure 5.** Percentage contribution of fungal families in lignocellulose degradation.



**Table 3.** List of fungal strains with their potential to degrade lignin.

Name of the Fungi	Percentage of Lignin Degradation	References
<i>Phanerochaete chrysosporium</i>	30 and 34.3	[47,48]
<i>Trametes versicolor</i>	22	[49]
<i>Pleurotus ostreatus</i>	41	[50]
<i>Phlebia</i> sp. MG-60	40.7	[51]
<i>Pleurotus eryngii</i>	63 and 75	[52]
<i>Lentinula edode</i> LE16	87.6	[53]
<i>Gloeophyllum trabeum</i>	16	[54]
<i>Ceriporiopsis subvermispora</i>	22	[55]
<i>Galactomyces geotrichum</i>	48	[56]
<i>Polyporus ostreiformis</i>	18.6	[57]
<i>Dichomytus squalens</i>	34.1	[58]
<i>Phlebia</i> sp. MG-60	40.7	[51]
<i>Fomitopsis pinicola</i>	32.4	[58]
<i>Fusarium</i> sp. 89, <i>Fusarium moniliforme</i>	33.5, 34.7	[59]

Apart from terrestrial fungi, some marine fungi such as the marine cnidarian fungus *Cladosporium cladosporioides* CBMAI 857, *Aspergillus sclerotiorum* CBMAI 849, and *Mucor racemosus* CBMAI 847 were able to synthesize ligninolytic enzymes. In the studies conducted by Bonugli-Santos et al., (2010) it was reported that the fungus *M. racemosus* CBMAI 847 produced the highest values for lignin peroxidase (75376.34 UI L<sup>-1</sup>) followed by Manganese peroxidase (4484.30 UI L<sup>-1</sup>), and Laccase (898.15 UI L<sup>-1</sup>). These enzymes are frequently referred to as lignolytic enzymes which play an important role in the degradation of lignin.

This figure clearly represents that, cellulose was highly depolymerized by the action of the ascomycetes and basidiomycetes fungal groups almost equally. Hemicellulose was significantly degraded by the ascomycetes group followed by basidiomycetes. In the case of lignin, the basidiomycetes are the major players followed by ascomycetes.

## 2.2. Significance of White Rot Fungi in Lignin Degradation

The white-rot fungus (mainly Basidiomycota) are key contributors to lignin depolymerization because of the presence of extracellular laccase and peroxidases, their presence assists in the degradation of lignocellulose, also known as Lignin Modifying Enzymes (LMEs) such as Laccases, Lignin Peroxidases, Manganese Peroxidases, and Versatile Peroxidases [60]. The white rot fungi are classified as simultaneous and selective degraders. These selective lignin degraders from white rot fungi are focused significantly in biotechnology applications in lignin elimination [61]. The depolymerization metabolites are heterogeneous aromatics that other microorganisms can use in the future [62]. White-rot fungi are capable of mineralizing lignin in soil, despite the soil's resistance to the process. The majority of the lignin in the soil is connected to humic compounds, yet white-rot fungi could mineralize lignin bound to humus [63]. *Phellinus pini*, *Ganoderma australe*, *Phlebia tremellosa*, and *Ceriporiopsis subvermispora* are white-rot fungi that break down only lignin and hemicellulose. Other strains, such as *Trametes versicolor*, *Phanerochaete chrysosporium*, *Irpex lacteus*, and *Heterobasidion annosum* can break down cellulose, hemicellulose, and lignin all at the same time [14]. Various enzymes that are detected from the white rot fungi are shown in the following Table 4.

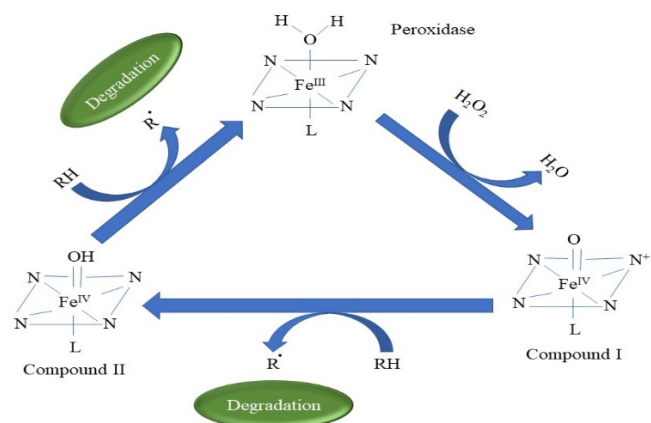


**Table 4.** Lignolytic Enzymes detected from white rot fungi.

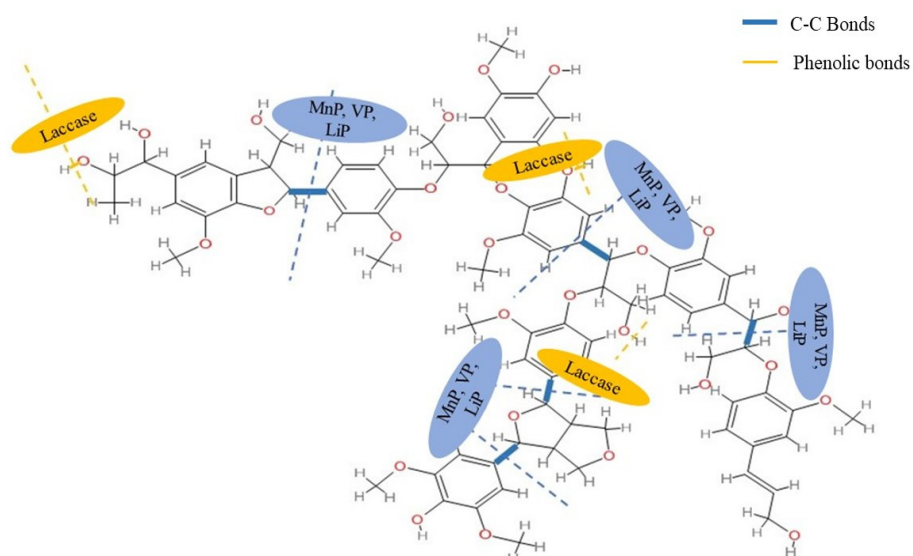
Name of the White Rot Fungi	Name of the Enzymes	References
<i>Artemisia biennis</i>	Laccase, Manganese peroxidase	[63]
<i>Hypoxylon fragiforme</i>	Lignin peroxidase, Manganese peroxidase	[64,65]
<i>Bjerkandera adusta</i>	Laccase, lignin peroxidase, Manganese peroxidase, Aryl alcohol oxidase	[63]
<i>Oxyporus latemarginatus</i>	Lignin peroxidase, Manganese peroxidase	[64]
<i>Dichomitus squalens</i>	Laccase, Manganese peroxidase	[63,66]
<i>Phanerochaete sordida</i>	Manganese peroxidase	[63]
<i>Phlebia. radiata</i>	Laccase, lignin peroxidase, Manganese peroxidase, Glyoxyl oxidase	[63,64,66]
<i>Phlebia brevispora</i>	Laccase, lignin peroxidase, Manganese peroxidase.	[64,66]
<i>Pleurotus ostreatus</i>	Laccase, Manganese peroxidase, Aryl alcohol oxidase	[63]
<i>Phlebia tremellosa</i>	Laccase, lignin peroxidase, Manganese peroxidase.	[64,66]
<i>Trametes hirsuta</i>	Laccase, Manganese peroxidase	[63]
<i>Ceriporiopsis subvermispora</i>	Laccase, Manganese peroxidase	[64,66]
<i>Trametes versicolor</i>	Laccase, lignin peroxidase, Manganese peroxidase, Aryl alcohol oxidase.	[63]

### 3. Fungal Enzymatic Depolymerization of Lignin

The lignin biological catabolism is a naturally occurring process, enabling the deconstruction of lignin through a wide range of enzymes under naturally optimized conditions. Their genomic sequencing exposes a diverse range of genes involved in lignin decomposition putting the parent family of basidiomycetes on the pedestal of the most preferred fungi for lignin-degrading biotechnological applications [67]. The technical challenge with this group of fungi is that they guard their secrets of enzyme production very strongly and most of them are still unknown to humans. There have been elusive results of heterologous production of basidiomycetes for rapid detections of enzymes. The simulation of the enzymatic degradation process is very complex and not very successful at an industrial scale. Figure 6 represents oxidation mechanism of peroxidase.

**Figure 6.** Oxidation mechanism of peroxidase.

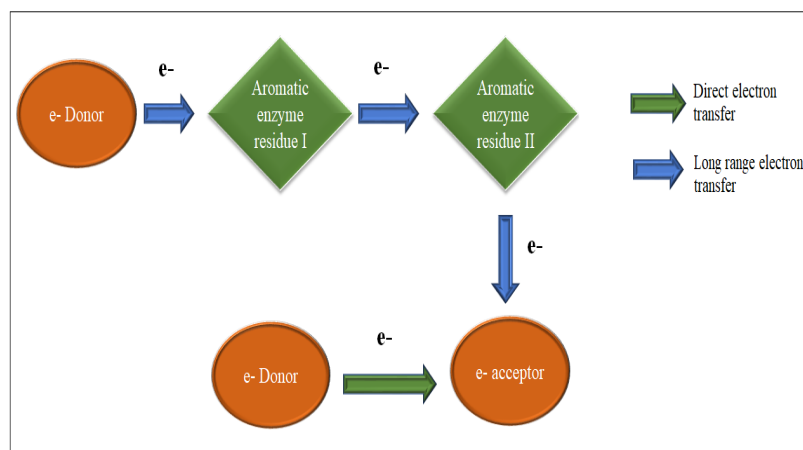
The redox potential of versatile peroxidase is largely unexplored and remains a persistent research question. An attempt of understanding the comprehensive functioning of lignocellulose degrading enzymes and the redox potential of versatile peroxidase is made through an illustration (Figure 7) explaining MnP, VP, LiP; their location, and action of degradation in lignocellulose. The role of lignolytic enzymes in lignin degradation are represented in Figure 7.



**Figure 7.** Role of lignolytic enzymes in lignin degradation.

Looking at the complexities involved, the *in vivo* or *in-cell* secretion of the degrading system is the best-fit option. This makes the microbial model organism, *Saccharomyces cerevisiae* as the obvious next choice for heterologous production of delignifying enzymes although the challenge of non-secretion of nature heme peroxidases still remains with this microbe. To address this problem, scientists consider using a plant-based expression for lignin-degrading heme peroxidase and plants natively produce this enzyme extracellularly for biosynthesis and morphogenesis of cell walls [68].

Basidiomycetes fungi are equipped with high redox potential to perform electron oxidation during lignin degradation. Fungal lignin peroxidases (LiP) resemble closely with other peroxidases in terms of their potential to oxidize recalcitrant substrates such as methoxybenzenes. Lignin peroxidases (LiP) generally use long-range electron transfer (LRET) to oxidize lignin compounds. Manganese peroxidases (MnP) use Mn (III) oxidation as a diffusible mediator whereas versatile peroxidases (VP) use both LRET and diffusible mediator as the mode of operation. The LRET mechanism (Figure 8) is an important and evolutionary adaptation in ligninolytic peroxidases because it allows electron abstraction from lignin, as lignin is thick and bulk, it restricts direct contact with activated cofactor at the protein's surface.

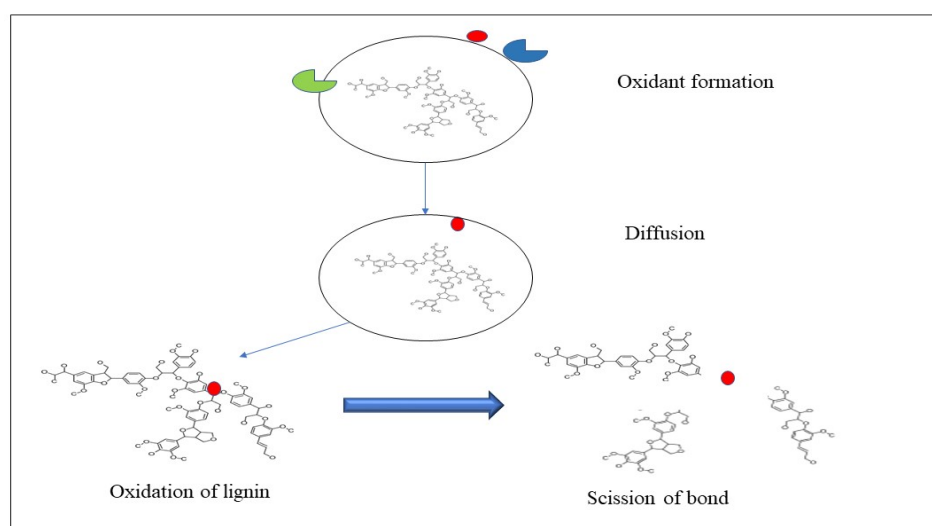


**Figure 8.** Shows the transport mechanism of electrons in direct electron transfer and long-range electron transfer (LRET).

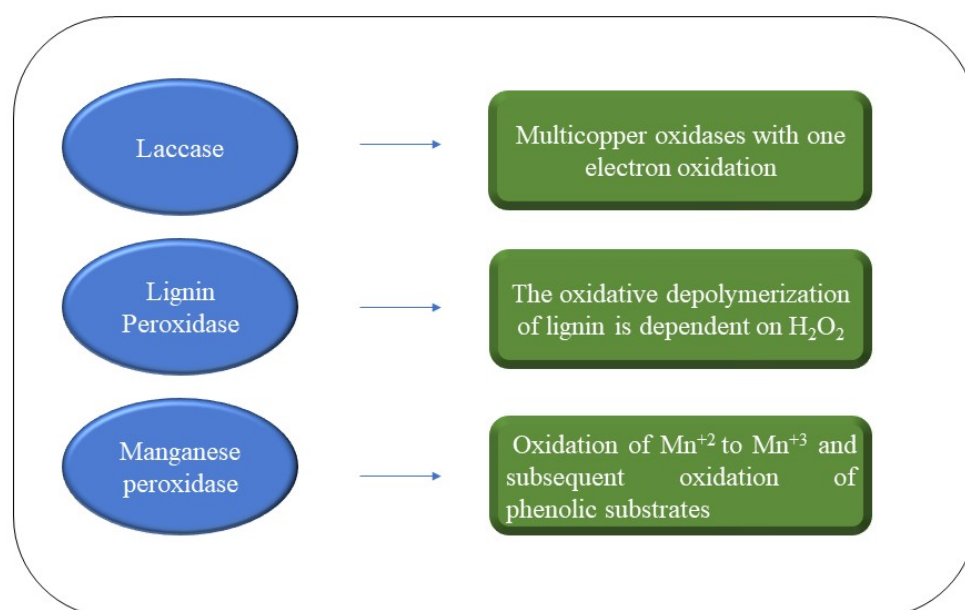
A better understanding of the genetic machinery of lignolytic enzyme-producing basidiomycetes is still in the infancy stage and requires more knowledge on the folding and processing of such enzymes. The model microbes have proven to be insufficient as they mostly lack the specialized cellular environment which is acting as a production site for the functional expression of lignolytic enzymes. The studies [68,69] also suggest the possibility of the existence of a more complex and specialized cellular machinery in basidiomycetes for addressing the oxidative stress resulting from peroxidase production.

### 3.1. The Mechanism Involved in Lignin Depolymerization

As shown in Figure 9, The first step involved in lignin degradation is to form radical mediators via enzymatic reaction. Later, this diffuses into the lignin substrate and then the oxidizing equivalent will be transferred to the polymer. The formation of the lignin-based radical, results in bond scission and ultimately leads to the depolymerization of lignin [70]. The oxidation mechanism of lignolytic enzymes is shown in Figure 10.



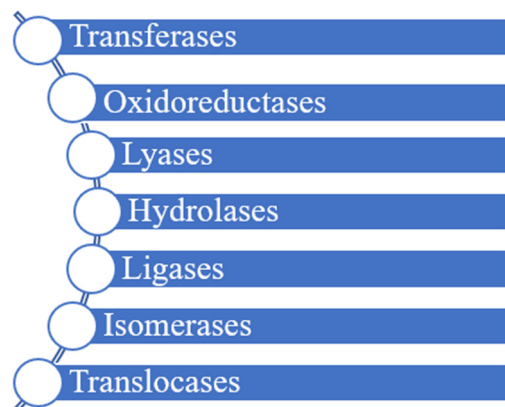
**Figure 9.** The mechanism involved in lignin depolymerization.



**Figure 10.** Oxidation mechanism of various lignolytic enzymes.

### 3.2. Role of Different Enzymes in the Fungal-Assisted Depolymerization Pathway

The mycelial growth system of fungi is responsible for the transportation of nutrients from distant host parts to the fungi. The key transported materials are nitrogen and iron to the lignocellulosic host which is the sole carbon source for fungi armed with extracellular hydrolytic and oxidative enzymes, fungi degrade lignin and open the phenyl rings [37]. The enzymes are classified into seven classes as shown in Figure 11.



**Figure 11.** Classes of Enzymes.

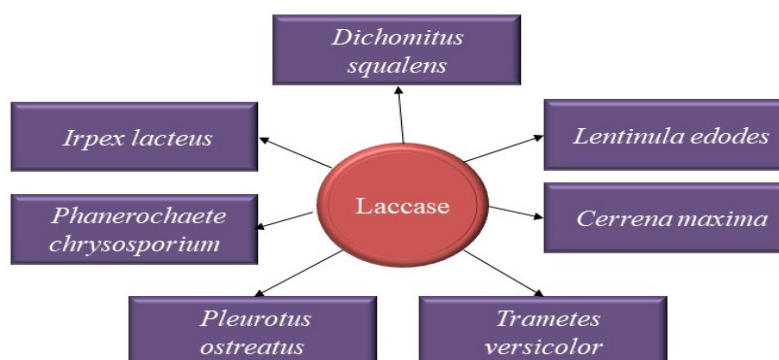
Apart from individual enzymes, the depolymerization of lignocellulose also occurs by the action of enzyme cocktails which can be understood as (free enzymes, each containing one/more catalytic domains) and cellulosomes (complex multienzyme complexes with many catalytic units). By peeling away pockets of cell wall lamellas, cellulosomes gain access to deeper lamella layers. Free enzymes that infiltrate the accessible walls destroy the accessible microfibrils synergistically. Cellulosomes are produced by *Ruminococcus*, *Clostridium*, *Bacteroides*, and *Acetivibrio* genera of mesophilic anaerobic bacteria [71].

Lignolytic enzymes such as Lignin Peroxidase (LiP), Laccase, Versatile Peroxidase, Dye-decolorizing Peroxidase (DyP) and Manganese Peroxidase (MnP) are all well-known lignin-degrading enzymes [72]. To take another step towards depolymerization into basic sugars Lytic Polysaccharide Monooxygenases (LPMOs), a revolutionary new generation microbial enzyme, is being considered a revolutionary tool and combining LPMOs with other glycolytic enzymes is effective in the conversion of agricultural waste to valuable compost [73].

Laccases enzymes produced by white-rot fungi are capable of oxidizing a variety of phenolic and nonphenolic compounds. Peroxidases are biocatalysts for the bioremediation of harmful environmental pollutants. Cellulases differ in their ability to hydrolyze the  $\beta$ -1,4-glycosidic links that join glucose units in cellulose fiber. Other enzymes play a critical role in oxidizing many primary alcohols such as glyoxylate oxidase. The details regarding lignolytic enzymes are as follows:

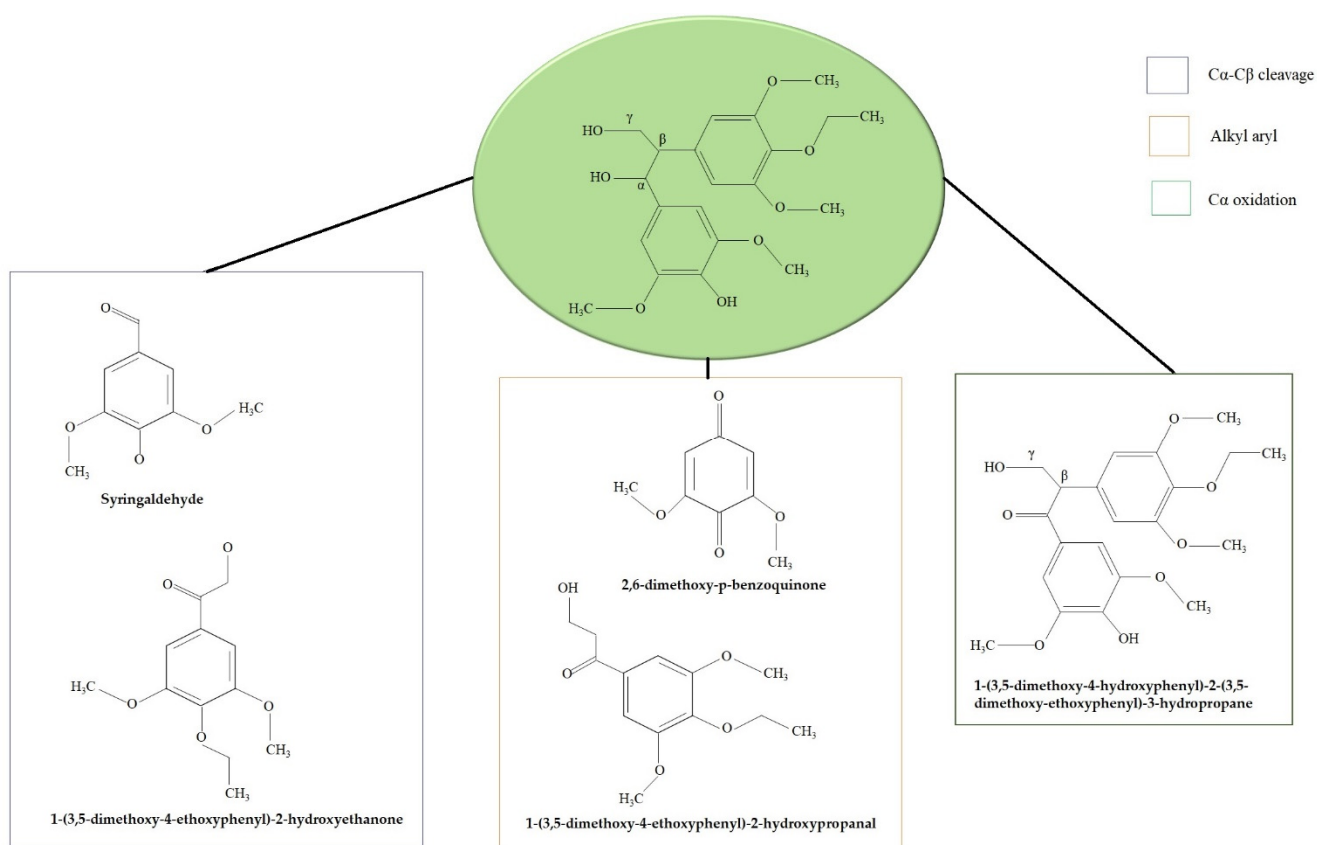
#### 3.2.1. Laccase

Laccase is a representative enzyme of the multicopper enzyme family laccases. Laccase-mediated reactions are linked to the generation of radicals. They can be aided by a class of tiny molecules known as redox mediators because ligninolytic enzymes are primarily released to deconstruct lignin / humic compounds. This transition is defined as cometabolic, which refers to the unintentional degradation of a contaminant through enzymes secreted during the microbial metabolism of some other substance [62]. The majority of ligninolytic fungus species generate at least one laccase isoenzyme as a constitutive product, and laccases predominate among ligninolytic enzymes in soil environments. They are useful for biotechnological applications for the reformation or immobilization of xenobiotic substances since they just need molecular oxygen for catalysis [74]. Some of the common laccase-producing fungi are represented in the following Figure 12.



**Figure 12.** Key fungal groups actively producing Laccase enzyme which predominates the lignolytic enzyme group.

The generic mechanism of lignin depolymerization by laccase enzyme is explained in detail. The C $\alpha$ -C $\beta$  cleavage, Alkyl aryl, and C $\alpha$  oxidation is the site of action of the laccase enzyme as illustrated in Figure 13. They reduce and form the products such as syringaldehyde and 1-(3,5-dimethoxy-4-ethoxyphenyl)-2-hydroxyethanone through C $\alpha$ -C $\beta$  cleavage, 1-(3,5-dimethoxy-4-ethoxyphenyl)-2-hydroxypropanal and 2,6-dimethoxy-p-benzoquinone through Alkyl aryl, 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(3,5-dimethoxy-ethoxyphenyl)-3-hydropropane via C $\alpha$  oxidation.

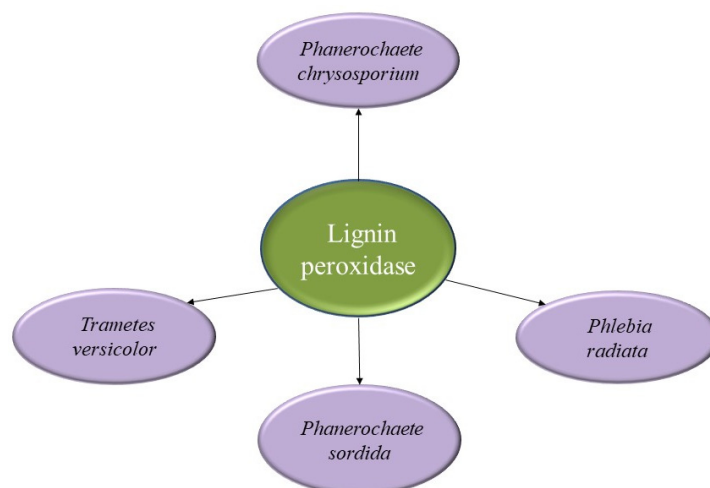


**Figure 13.** The mechanism involved in lignin depolymerization by laccase.

### 3.2.2. Lignin Peroxidase

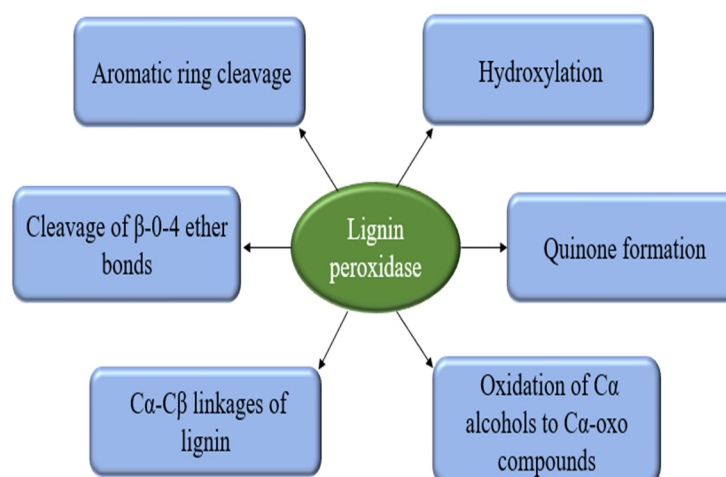
Lignin Peroxidase was the first ligninolytic enzyme identified from *Phanerochaete chrysosporium*, a white-rot fungus [75]. The non-phenolic lignin units are known to be degraded effectively by the LiP. They promote the hydrolysis process by targeting the

lignin layer found in lignocellulosic biomass early in the process. LiP catalyzes oxidative lignin breakdown in the presence of  $H_2O_2$ . The oxidation of lignin involves electron transport, opening aromatic rings, and the non-catalytic breakage of multiple bonds. These enzymes are considered the strongest biocatalyst in bioremediation [76]. Lignin peroxidases are mainly obtained from the fungal species represented in the following Figure 14.



**Figure 14.** Some of the fungi that secrete lignin peroxidase for lignin degradation.

As shown in Figure 15, the LiP can catalyze several reactions such as  $\beta$ -0-4 ether bonds cleavage and  $C\alpha$ - $C\beta$  linkages. These two cleavages play a very significant role in lignin depolymerization. Moreover, they also catalyze the oxidation of  $C\alpha$  alcohols to  $C\alpha$ -oxo compounds, hydroxylation aromatic ring cleavages, and quinone formation. The quinone formation is a significant step as they polymerize to form humic substances as shown in Figure 3. The intermediates involved in these catalytic reactions are cation radicals. Generally, LiP is considered a highly efficient enzyme, having the potential to degrade even the diluted lignin solutions but due to the involvement of the radicals, they can immediately repolymerize [77].



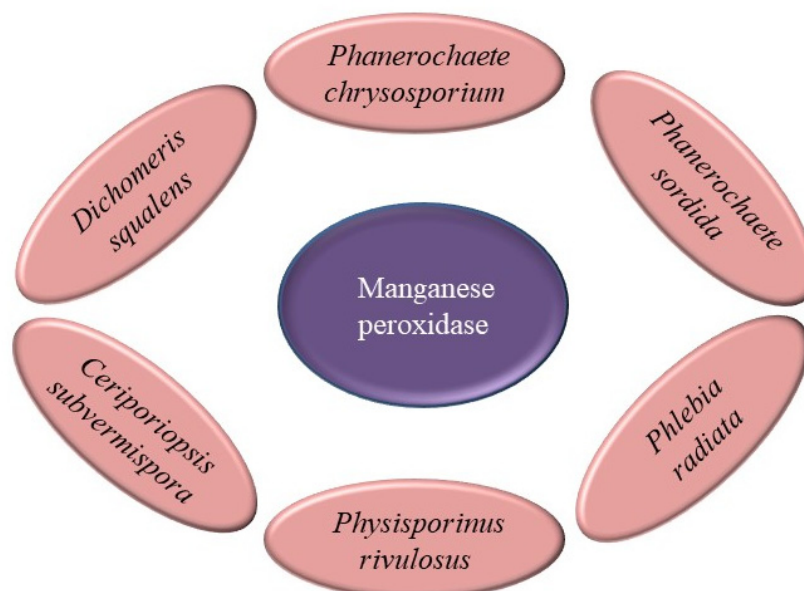
**Figure 15.** Different catalytic actions by LiP.

### 3.2.3. Manganese Peroxidase

Manganese Peroxidase (MnP) has been proposed as a phenolic structure degrader. Similar to an electron donor in MnP,  $Mn^{2+}$  is oxidized to  $Mn^{3+}$  and becomes stable as a result of chelation with dicarboxylic acids. Chelated  $Mn^{3+}$  then works as redox mediator, breaking down non phenolic units [78].



It may oxidise  $Mn^{2+}$  to  $Mn^{3+}$  by diffusing off its surface and oxidising phenolic substrates such as lignin combinations and organic pollutants. By oxidising  $Mn^{2+}$ , which is abundant in wood, MnP has evolved the potential to fracture minor phenolic lignin. Furthermore, MnP cannot oxidise non-phenolic chemicals, which have a higher resistance to oxidation, it accounts for up to 90% of lignin polymer [76]. Manganese peroxidases mainly obtained from the fungal species are represented in the following Figure 16.



**Figure 16.** Some of the fungi that secrete Manganese peroxidase.

#### 4. Protein Engineering Approaches towards Improving the Efficiency of Enzymes

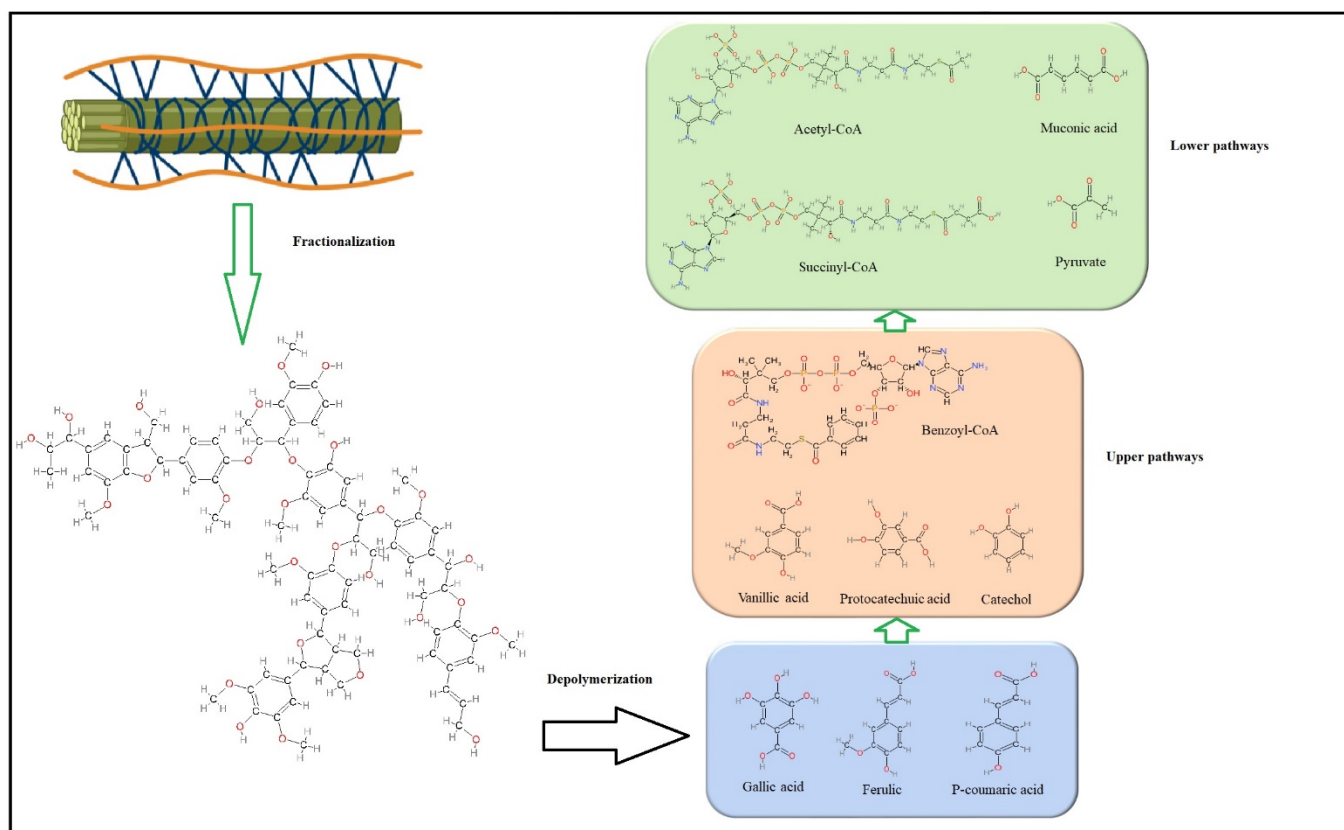
The majority of the enzymes that are utilized are either from cultured microorganisms or metagenomic methods. They need to work well for a low price and within the parameters of the manufacturing process that have been specified. Industrial processes must be modified to adapt to these subpar biocatalysts because the enzymes used in industry are not working as well as they should. In this specific circumstance, one of the primary choices to return what is going on is to synthetically or hereditarily alter proteins to work on their properties. The two main approaches that can be used to carry out engineering are directed evolution and rational design. In this method, the protein-coding sequence is changed (through insertions, duplications, mutations, deletions, recombinations, and so on) to produce a collection of unique proteins; Directed evolution performs a faster simulation of natural selection. After that, these variations are evaluated for at least one trait that helped them advance to the next level. The rational design makes use of computational tools and prior knowledge of the structure of proteins to deliberately design novel proteins. Both rational design and directed evolution approaches to protein engineering are used to enhance the protein properties of enzymes implicated in (a) depolymerization and (b) lignocellulosic material's fermentation for the production of biofuels or fine chemicals [79]. The following paragraph provides recent examples of the studies.

Recent research has focused on the implementation of directed evolution methods to bacterial Dyp-type peroxidase enzymes., it is also reported that an error-prone polymerase chain reaction was used to engineer *P. putida* DyP, resulting in a mutant enzyme with three mutations on its surface—E188K, A142V, and H12V [80]. Rahmanpour and others reported that focused libraries were used around the active site of *P. fluorescens* Dyp1B, which resulted in a 7–8 fold increase in the  $K_{cat}/K_M$  for 2,4-dichlorophenol. These mutations also shift the optimal pH to 8.5. Additionally, the mutation H169L was found to increase polymeric lignin product release [81].



## 5. Hybrid Biochemical Routes of Lignin Valorisation

In general, the initial step involved to break lignocellulose is fractionalization and then depolymerization to give ferulic acid, p-coumaric acid, gallic acid etc. The next step is the upper path way which yields vanillic acid, protocatechuic acid, catechol, and benzoyl-CoA and the lower pathway gives muconic acid, pyruvate acetyl-CoA, succinyl-CoA as represented in Figure 17 [82].



**Figure 17.** Depolymerization and pathways of lignin.

### 5.1. Fungal Depolymerization Pathways

Lignin having lower solubility in the aqueous phase is one of the major challenges for the enzymatic depolymerization of lignin. Lignin depolymerization is an end-step process and there are several prerequisites for the depolymerization such as fractionation from the lignocellulose with the help of different pre-treatment processes such as acidic, alkaline, and hydrothermal, etc. But again, these methods have a major limitation of the low amount of lignin removal so the process becomes very cost-intensive for pretreating and final degrading. To make the process industrially viable and sustainable, the biological resources for lignin degradation are offering a silver lining [83].

Biological depolymerisation is extensively researched and it was found that various fungi are able to produce several effective oxidative enzymes such as Laccases, Aryl Alcohol Oxidases, Manganese Peroxidases (MnP), Lignin peroxidase (LiP), Dye Decolourizing Peroxidase, etc. [52]. The lignin depolymerization by fungal metabolisms method and their isolated enzymes are shown in Figure 18.

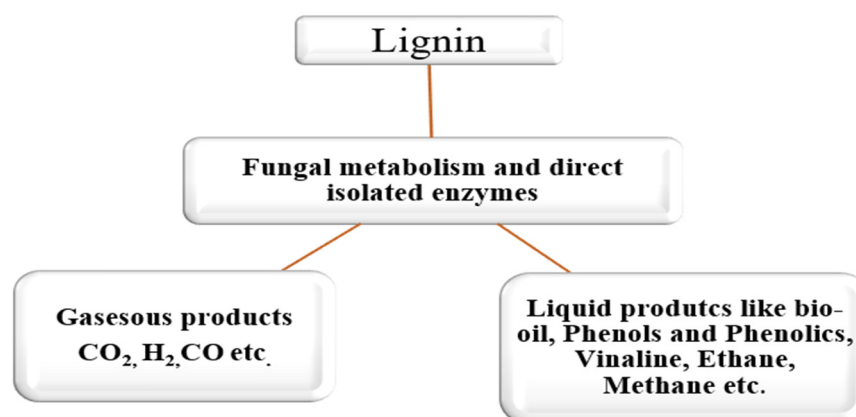


Figure 18. Lignin depolymerization by fungal metabolisms method and their isolated enzymes.

Other than white rot, the brown rot fungi degrade the lignin by the Fenton oxidation process or by producing hydroxyl radicals. The entire process of redox cycling is initiated with the oxidation of Quinone to hydroxyquinone which converts  $\text{Fe}^{+3}$  into  $\text{Fe}^{+2}$  which in the end reacts with hydrogen peroxide to produce hydroxyl radicals.

On the other hand, very limited information is available about the mechanisms of the soft rot fungi which were originated from the white rot fungi and are able to degrade the lignin content by targeting higher moisture and lower lignin-containing materials and Syringyl units [84]. Some of the soft rot fungi are able to rapidly degrade the last stage products of the lignin degradation pathways such as vanillic acid and phenols which made them suitable components as co-culture or multi-organisms mediated lignin depolymerization [85]. The steps involved in lignin depolymerization are shown in Figure 19.

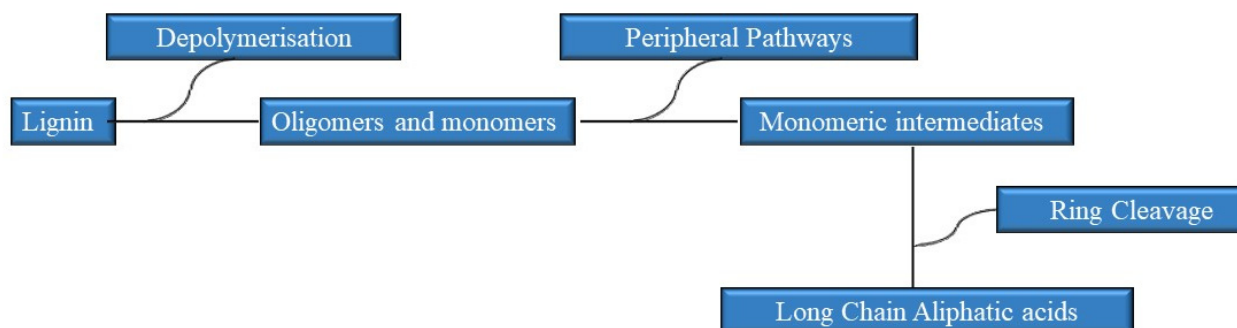


Figure 19. Step-wise process of the depolymerization of Lignin [86].

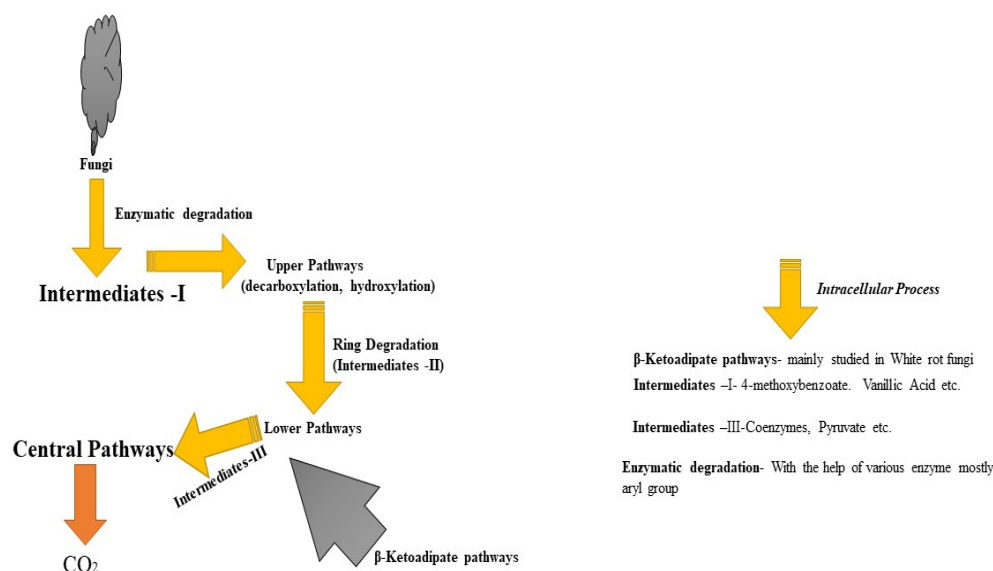
#### 5.1.1. Model Organism

Traditionally, the white rot fungi *Phanerochaete chrysosporium* is regarded as the model organism for the studies related to lignin polymerization. The microorganisms produce multiples isozymes of the MnP and Lip which may be effective in lignin depolymerization but one of the major limitations of this species is the production of laccases. So, it includes advantages in the last stage of the degradation in case of the nitrogen made available by the other organisms [7].

#### 5.1.2. Major Pathways

Lignin is the second most abundant biopolymer in the entire earth's environment, the catabolic pathways (aromatic) are the most efficient manner used by microorganisms to degrade the lignin content for their energy requirement fulfillment (energy production). So, the entire catabolic pathway has three main steps and among them, depolymerization is the first one and the other two are aromatic degradation and product biosynthesis. So, aromatic catabolism is regarded as the "Upper pathways" which produce several intermediates

for “Peripheral Pathways” or “Lower Pathways”), e.g. Catechol. The intermediate of the upper pathways further degraded by the one of the key pathways  $\beta$ -ketoacid pathway. This pathway is intensively studied in the white rot fungi. The end product of the  $\beta$ -ketoacid pathway functions as the intermediated for the Tricarboxylic Acid Cycle (TCA) which is central to the entire process and called the central pathway [87]. The schematic representation of the fungal biological pathways in depolymerization and degradation of lignin is shown in Figure 20.



**Figure 20.** A hypothetical schematic representation of the biological pathways (fungal) involved in the depolymerization and degradation of the lignin [88].

### 5.2. Current Research and Future Trends

As described, above the degradation of lignin is a complex process and due to the low solubility of lignin in the aqueous phase it becomes more challenging and depolymerisation is the starting phase of the degradation so researchers are trying to use an enzyme multi-enzyme system for better results. Co-culture system is also used in the degradation of lignin. Very recently consortium of white rot fungi was used for the enhanced degradation of lignin [89]. Table 5. having information about the impact of the co-culture of white rot fungi on the monoculture system.

**Table 5.** Co-culture system with improved lignin degradation.

Dual Culture or Consortium	Lignin Degradation Percentage	References
<i>Anthracophyllum discolor</i> + <i>Stereum hirsutum</i>	94.1	[90]
<i>Lenzites betulina</i> + <i>Trametes versicolor</i>	More than 40 % enhancement than monoculture	[89]
<i>Coprinus comatus</i> + <i>Trichoderma reesei</i>	66.5	[91]
<i>Daedalea flavida</i> MTCC 145 + <i>Phlebia radiata</i> MTCC 2791	36.29	[92]
<i>Phanerochaete chrysosporium</i> + <i>Irpex lacteus</i> CD2	26.4	[93]
<i>Phanerochaete chrysosporium</i> + <i>Trichoderma viride</i>	26.38	[94]
<i>Lenzites betulinus</i> + <i>Trametes Orientalis</i> + <i>Trametes Velutina</i>	58	[95]

## 6. Products from Lignin Valorization

The excess lignin extracted from lignocellulosic biomass provides a sustainable source of a range of aromatic compounds that may be further converted into high-value chemicals [96]. Additionally, the lignocellulosic biorefinery model may become financially viable thanks to lignin depolymerization, which could replace the use of aromatic materials derived from petrochemicals. Among the high-value aromatic chemicals that can be de-

rived from lignin are vanillin, P-hydroxybenzoic acid, Muconic acid, pyrogallol, benzene, toluene, and xylene. Lignin can also be transformed into bulk aromatic chemicals by aerobic microorganisms through fermentation and transformation.

Biotechnological lignin valorization by enzymatic conversion/degradation is a novel cost- and energy-efficient sustainable method. However, biotechnological conversion of lignin is difficult because native organisms that are capable of converting lignin into economically viable value-added product portfolios are rarely adapted to industrial settings. Redesigning the metabolic pathways is absolutely necessary to speed up the rate of conversion of these aromatic derivatives from lignocellulosic into bio-based products [97].

Lignin biodegradation by microbes has also been studied as a crucial pretreatment step in the production of biodiesel from cellulose. Zhang et al. state that Lipids that can be used as a feedstock for the production of biodiesel can also be directly produced by lignin degradation. Sonoki et al. used as substrates mixtures of vanillic acid, 4-hydroxybenzoic acid, and syringic acid, lignin model compounds [98]. The KT2440 strains of *Pseudomonas putida* and *Sphingobium* sp. SYK6 bacteria break down lignin and its derivatives to produce cis, cis-muconic acid, a precursor to several industrial polymers [99].

Thanks to advances in metabolic engineering and synthetic biology, it is now possible to produce polyhydroxyalkanoates (PHAs) from microorganisms utilizing using lignin as a substrate at a price that is competitive. Recent years have seen Brown et al., By communicating the Phap1 phasin (phaP1) qualities of the *Cupriavidus necator* H16 bacterial model in *R. palustris*, the *Rhodospseudomonas palustris* CGA009 bacterium aims to overproduce a PHA known as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) from the breakdown product of lignin, p-coumarate. According to their findings, *R. palustris* heterologous phasin expression led to PHBV aerobic bioplastic production (0.7 g/L) that was greater than the wild-type anaerobic production (0.41 g/L) [100].

Microorganisms have the ability to transform lignin or substances derived from lignin into lipids, which are regarded as a sustainable material for the production of biodiesel. Microbial species of oleaginous may metabolize aromatic lignin into lipids by utilizing it as a nutritional substrate [101].

The utilization of biomass to produce high-value biofuels such as biogas and the valorization of the lignin stream for the production of cellulosic ethanol could potentially boost the profitability of biomass-based biorefineries. The large quantities of lignin-rich residues produced by lignocellulosic biomass (LCB) pretreatment can be converted into biogas through anaerobic digestion (AD), thereby increasing the energy carrier output of biorefinery plants. Because of its delayed decomposition and nutrient release, lignin is a great source material for soil-enriching fertilizers such as phosphate fertilizers, chelated micro-fertilizers, and slow-release nitrogen fertilizers [102]. After being changed with various elements, lignin-derived fertilizers display excellent fertilizer efficiency, improved biological activity, long-term stability, economical, anti-leaching qualities, and low pollution [103].

## 7. Challenges in Lignin Biomass Conversion through Microbes

Due to the variation of lignin that results in combinations of products, isolating and purification of the distinct components for use in future applications will be challenging and less economically feasible. However, in order to identify the most efficient technologies for separating lignin from raw biomass and depolymerizing isolated lignin into value-added products, a comprehensive understanding of every conversion process in a lignin biorefinery and the life cycle of lignin-derived products from upstream to downstream is required [104].

Although enzymes have a strong ability to valorize lignin, it is evident that significant breakthroughs to get around multiple important obstacles are required before enzyme lignin valorization can be used in the real world. First, the commercialization of the enzyme-based degradation process of lignin has been hampered by the enzymes' low productivities. Only in sufficient quantities enzymes are produced to provide a fundamental understanding of the process of degradation. However, there is insufficient supply for extensive commercial

applications. Overexpression of a few proteins may be beneficial, but it can also result in the production of proteins with little or no activity or none of the desired proteins.

Due to the difficulty of protein expression and genetic modifications, as well as the absence of useful genetic information for fungal enzymes, industrialization of fungal enzyme degradation is also challenging. Despite tremendous advancements, the role of mediators in the enzymatic degradation pathway remains unknown. Additional concerns include toxicity of the mediators, recycling, and cost.

More binding sites can be added to enzymes with genetic modification tools to significantly increase their activities and efficiency, but this may have an effect on enzyme yield. To put it succinctly, extensive research is required to clarify and confirm the relationship between the types of mutations and the expression level. Because re-polymerization of phenolic compounds happens often when the lower molecular weight compounds are not eliminated, the removal of phenolic compounds generated during the degradation process remains a stumbling block in large-scale lignin degradation. In addition to degrading lignin, MnP may polymerize guaiacol, o-cresol, and 2,6-dimethoxyphenol [105]. Some of the other highlights are listed as follows:

- There is a gap in understanding the synergistic action of the fungal enzymes and the enhancement of the delignification efficiency.
- There is a need of applying molecular-level and genetical biotechnologies to achieve an industrial level of application
- Biosafety Issues-Fungal enzymes allergy for humankind.
- The substrate bioavailability of the lignin and rate of conversion is the main challenge. Moreover, industrial applications and process scale-up still have some challenges and need to be addressed [106].
- Single cultures are ineffective in breaking down the lignocellulose components. Delignification cannot be achieved by a single species, as studies have revealed that in nature, lignin depolymerization is achieved by the combined efforts of many microorganisms under aerobic and anaerobic environments [107].
- The secretion of enzymes from bacteria necessitates a higher level of specificity than other processes. The construction and research of bacterial enzyme complexes are more complicated [108].

## 8. Way Forward and Future Prospects

The search for stable and strong inherent cellulolytic enzymes from varied and distinct environments, all of which are definitely of microbial origin, might be a fundamental strategy for the successful exploitation of readily accessible cellulosic substrates, particularly for bio-refineries. In the future, a wide range of molecular and genetic tools and techniques, such as strain enhancement by mutagenesis, genetic engineering, structure-guided recombination, heterologous gene expression, homologous, and so on, must be used to improve an in-depth understanding of the potential source of cellulolytic enzymes and their efficient production [109].

Due to ongoing advancements in gene synthesis, deep sequencing, continuous evolution, and ultra-high-throughput screening methods, new biocatalysts are already being discovered experimentally. As well as being more qualified for bioprocesses or more dynamic, these clever chemicals should be efficient for modern use. Research into microbial enzymes that can convert biomass more effectively also requires the ability to engineer enzymes within the context of a genome. At the present time, the packed regularly interspaced short palindromic reiterate/related protein 9 (CRISPR/Cas9) system is the most reassuring contraption for genome adjusting. CRISPR/Cas9-based tools for directed evolution and rational design may become an appealing option for the quick and efficient protein engineering of enzymes on industrial strains' chromosomes [79].

Lignin isolation and depolymerization processes also have an impact on the composition of depolymerized lignin feedstocks. Regardless of the feedstock composition, a perfect microbe would efficiently catabolize all lignin-derived compounds. However, such



a microbe has not yet been discovered or created. More specifically, we are able to select microbes based on the feedstock's composition using feedstock-tailored microbes or microbial systems. Utilizing and engineering previously characterized microbes that are suitable for another depolymerized lignin feedstock that is rich in the compounds these employed microbes are known to efficiently utilize can be used to create these "tailor-made" microbial systems. It is possible to discover new microbes that effectively utilize the majority of the compounds in a particular feedstock of depolymerized lignin [110].

- Microbial enzymes via fungi can be produced quicker, cost-effective and is scalable, hence it is crucial in nourishing life and is sustainable
- Continuous search for fungal novel enzymes can lead to numerous nutritional value and health benefits for mankind
- The diverse enzymatic actions of fungi such as mushrooms can leverage low-cost agricultural production systems.
- Exploring of synthetic biology for improving the lignin degradation process.
- Food fermentation can be naturally performed by fungi enzymes whose application will enhance the preservation and shelf life of foods without affecting the characteristics of the organoleptic and nutritional content of foods [111]
- Treatment of lignocellulosic agricultural waste by fungi that are naturally abundant in diverse enzymes can valorize cellulosic material into valuable industrial bioproducts such as biofuels.
- Antioxidants are essential for healthy living and fungi have the capacity to produce antioxidant metabolites such as phenolics and flavonoids.
- Industrial applications of the fungal enzymes represent a sustainable, eco-friendly, and energy-saving solution for many environmental and quality aspects compared to the currently applied conventional chemical approaches.

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