Biotechnology Reports 39 (2023) e00809

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Research Article

A high throughput screening process and quick isolation of novel lignin-degrading microbes from large number of natural biomasses

Nadia Sufdar Ali [a,b], Fang Huang [b], Wensheng Qin [a], Trent Chunzhong Yang, PhD [b,1,*]

[a] Department of Biology, Lakehead University, Thunder Bay, ON, Canada
[b] Aquatic and Crop Resource Development Research Centre, National Research Council, Ottawa, ON, Canada

ARTICLE INFO

Keywords:
High throughput screening
Lignin degradation
Laccase
β-glucanase
Xylanase

ABSTRACT

High throughput screening approaches can significantly speed up the identification of novel enzymes from natural microbial consortiums. A two-step high throughput screening process was proposed and explored to screen lignin-degrading microorganisms. By employing this modified culture enrichment method and screening based on enzyme activity, a total of 82 bacterial and 46 fungal strains were isolated from fifty decayed wood samples (100 liquid cultures) collected from the banks of the Ottawa River in Canada. Among them, ten bacterial and five fungal strains were selected and identified based on their high laccase activities by 16S rDNA and ITS gene sequencing, respectively. The study identified bacterial strains from various genera including *Serratia, Enterobacter, Raoultella*, and *Bacillus*, along with fungal counterparts including *Mucor, Trametes, Conifera* and *Aspergillus*. Moreover, *Aspergillus sydowii* (AORF21), *Mucor sp*. (AORF43), *Trametes versicolor* (AORF3) and *Enterobacter* sp. (AORB55) exhibited xylanase and *β*- glucanase activities in addition to laccase production. The proposed approach allowed for the quick identification of promising consortia and enhanced the chance of isolating desired strains based on desired enzyme activities. This method is not limited to lignocellulose and lignin-degrading microorganisms but can be applied to identify novel microbial strains and enzymes from different natural samples.

1. Introduction

Novel and innovative screening approaches are uncovering the existence of a wide range of previously unknown enzyme activities from active microbial communities distributed in nature. Biomass conversion utilizes microorganisms and their enzymes, such as cellulases, xylanases and ligninases, to transform lignocellulose into platform chemicals which can then be converted to various products, including biofuels, chemicals, and materials. Microorganisms that can effectively break down biomass in high yields are vital for achieving sustainable and economically viable production of value-added products from lignocellulosic feedstock [1,2].

Lignin is an abundant natural phenolic polymer with immense potential to be valorized into high-value bioproducts after degradation into short oligomers or monomeric subunits. More so, biological and enzymatic degradation of lignin has the potential to advance sustainable green biotechnology as a highly selective and environmentally benign substitute for conventional fossil-based fuel production [3–5]. Indeed, adequate removal of lignin from green waste, a vital byproduct in the paper industry and biofuel manufacture, occupies the center stage in lignin biodegradation and bioremediation initiatives [6]. In plant cell walls, cellulose, hemicellulose, and lignin are united in a tight embrace, and denuding them of lignin provides microbial consortia with larger accessibility for cellulose bioprocessing. The lignin separated from woody biomass is converted into target compounds like vanillin, polyhydroxyalkanoates, lipids, and cis-muconic acid, rendering it a promising feedstock. However, the structural complexity of lignin and expensive enzymes are the two main obstacles that reduce the process' efficiency and make it unprofitable [7].

Microbes that break down lignin can produce a range of enzyme combinations comprising several types of lignin-degrading enzymes, such as laccases, manganese peroxidases, and lignin peroxidases [8]. Proficient lignin degradation can be achieved by the concerted action of multiple enzymes that work in cascade reactions which enhance the saccharification of polysaccharides of lignocellulosic biomass. Screening of microorganisms with significant lignin degradation ability allows the

* Corresponding author.
E-mail addresses: wqin@lakeheadu.ca (W. Qin), trentczy@yahoo.com (T.C. Yang).
[1] Current address: BioWise Technologies Inc., Ottawa, ON, Canada.

https://doi.org/10.1016/j.btre.2023.e00809
Received 15 March 2023; Received in revised form 14 July 2023; Accepted 26 July 2023
Available online 28 July 2023

opportunity to discover and characterize novel enzymes that potentially allow low-cost biological processes. However, discovering lignin-degrading microorganisms with a high performance largely depends on the screening strategy. A variety of microbial screening approaches and strategies were employed and adapted to identify novel enzymes for specific functionalities and targets [9–12]. Culture-dependent screening strategies mainly utilize a culture enrichment method which involves growing microorganisms in a cultivating medium with lignin as the carbon source, and the screening is solely activity based without relying on the prior knowledge of the enzyme. After the culture enrichment process, the most common practice is to perform a qualitative screening using an agar plate containing dyes resembling lignin fragments, followed by a quantitative screening of selected microbes based on activity [13–16,].

The agar plate screening method is laborious, time-consuming, has low throughput, and lacks precision as it may not accurately detect the target enzyme. Submerged screening methods, on the other hand, offer better control over the culture conditions, which can lead to higher enzyme production. They also allow the possibility for high throughput (HTP) screening of a vast number of samples simultaneously. Additionally, through the use of specific assay conditions or detection methods, the target enzymes can be identified with better specificity and sensitivity compared to agar plate screening methods. Moreover, screening in liquid media also mimics industrial submerged culture conditions, thus allowing the identification of potential microbes and enzymes for industrial applications [17].

There are no studies reported thus far on the HTP screening process of a large number of natural biomasses based on liquid culture and specific enzyme activities. This study aimed to 1) develop an HTP process that speeds up the identification of efficient lignin-degrading consortia, requiring fewer iterations and labor and facilitating the quick isolation of a large number of efficient microbial strains; 2) application of the developed process in practical isolation of new ligninolytic strains from natural biomasses.

In the preliminary screening step, decomposed wood samples were obtained from the Ottawa River banks. Each sample was separately incubated using an enriched culture approach, and the total laccase activities of each culture were quantitatively screened on 96-well plates. This initial step allows quick HTP screening of a vast number of samples. For secondary screening, the majority of samples were rejected due to low or negative laccase activities, and only a small number of samples with high laccase activities were selected for the identification of individual strains with high enzyme activities. This technique significantly reduced the time and labor to go through a large number of samples, meanwhile increasing the success rate of identifying new bacterial and fungal strains with superior lignin-degrading ability. More interestingly, some lignin-consuming strains also exhibited significant xylanase and β-glucanase activities, suggesting that this technique may enhance the opportunity to identify super strains that express multiple enzymes simultaneously. In addition, the proposed strategy can be easily modified and adapted to isolate new strains based on different enzyme activities.

2. Materials and methods

2.1. Chemicals

Czapek dox broth (CDB), 2,6 Di methoxy phenol (DMP), Kraft lignin, Dinitrosalicylic acid, Glucose, Xylose, Xylan (Birchwood, EC. No: 232-760-6, Sigma), β- glucan (Barley, Cat. No: P-BGBL, Megazyme).

2.2. Sampling

The samples used in the study were long-decomposed logs ($n = 50$) randomly collected from a densely forested area near Ottawa river, Canada (geographical coordinates: 45° 26′ 59.7″N, 75° 41′ 23.3″W) for screening of potential lignin-degrading microorganisms. The samples were obtained in sealed sterile sampling containers and stored in the refrigerator at 4 °C.

2.3. Primary screening by culture enrichment method

Primary screening is carried out to identify wood samples with high laccase activity. Briefly, 0.5 g of each wood sample was inoculated onto two sets of 50 ml Czapek dox medium with 0.1% kraft lignin (referred to as L-CDB), (Kraft 98%, Sigma Aldrich, MW>28,000) pH 7.3 ± 0.3, for bacterial and fungal isolation, separately. The fungal culture medium was made selective by adding 50 ppm chloramphenicol to suppress bacterial overgrowth and incubated for two weeks. Similarly, with bacterial cultures, 100 ppm cycloheximide was incorporated in the medium to inhibit fungal overgrowth for 7 days of incubation [18]. Cultures were incubated at 28 °C with 125 rpm shaking under aerobic conditions. After incubation, broth cultures were centrifuged at 13,000 rpm at 4 °C for 5 min, and the supernatant was then treated as the crude enzyme, and laccase activity was measured spectrophotometrically.

2.4. Secondary screening for potential isolates

Based on the primary screening, the samples which showed high laccase activity was selected to validate potential laccase-producing microorganisms. In all cases, 1 ml broth culture of each bacterial samples was serially diluted up to 10^{-6} and plated on Luria Bertani (LB) agar plates (containing g L^{-1} of distilled water in: 10.0 g peptone, 5.0 g sodium chloride, 5.0 g yeast extract and 15.0 g agar). Similarly, fungal samples were plated on Czapek Dox Agar (CDA) plates (containing g L^{-1} of distilled water in: 30.0 Sodium Nitrate, 3.0 Magnesium Sulfate, 0.5 Potassium Chloride, 0.5 Potassium Phosphate Dibasic, 1.0 Ferrous Sulfate and 15.0 g agar) to obtain morphologically distinct pure colonies (see Supplementary Figure 1. This included 82 pure bacterial cultures and 46 fungal isolates, which were further inoculated individually on 50 ml L-CDB under the previously mentioned culture conditions to measure laccase activity quantitatively at 72 h for bacterial strains as the optimum laccase production of most bacterium ranges 24 h – 96 h [19,20] and sixth day for fungal strains [21,22]. The colonies which grew well in culture media and showed the highest laccase activity ($\geq$ 20 U/L for bacteria; $\geq$ 30 U/L for fungi) were selected for molecular identification and preserved at 4 °C for further studies. The total protein amount was measured using the Bio-Rad protein assay reagent, USA, and bovine serum albumin (BSA) as the standard protein, following Bradford's method. Time course analysis was also performed concurrently, to determine the optimal incubation period for laccase production by the selected isolates.

2.5. Sequence-based identification of ligninolytic microorganisms

To identify bacterial strains, genomic DNA was obtained from the samples using bacterial DNA isolation kit (Bio Basic, Canada). Universal eubacterial primers, 27F (5'AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3'), as described by [23], were used to amplify the 16S rRNA gene. The PCR reactions were conducted in a Bio-Rad T-100 Thermal cycler, with 35 cycles typically used to amplify the 16S rRNA gene after the initial denaturation at 95 °C for 2 min, followed by denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 2 min, and final extension at 72 °C for 15 min.

To identify fungal strains, pure mycelium was collected from each strain and fungal genomic DNA was isolated using a fungal DNA isolation kit (Bio Basic, Canada) with liquid nitrogen. The internal transcribed spacer (ITS) gene sequencing analysis was used to analyze the ITS1-5.8S-ITS2 genomic region of each fungal isolate. The for- ward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the ITS region

of the genomic DNA, as described by [24]. For the fungal PCR, the amplification process involved an initial denaturation step of 5 min at 95 °C, followed by 35 cycles of amplification at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and then a final extension of 10 min at 72 °C.

The PCR products were then resolved on a 1% agarose gel and purified using a Qiagen quick gel extraction kit. The purified PCR product was sequenced by Eurofins Genomics, Canada for 16S rDNA and ITS gene sequencing analysis for bacteria and fungi respectively. Multiple sequence alignment analysis of 10 type strains that showed the highest identity and similarity to the bacterial isolate's sequence was performed using MEGA X program. The evolutionary history was inferred using the neighbor-joining (NJ) criterion, and a bootstrap analysis was performed using 1000 pseudo-replicates.

2.6. Laccase enzyme activity assay

Laccase activity assay was performed spectrophotometrically using 2,6 Di methoxy phenol as a substrate according to the method of [25]). The centrifuged culture supernatant from bacterial and fungal cultures after 13,000 rpm for 5 min was used as the crude enzyme. The assay mixture contained sodium acetate buffer (0.1 M, pH 5), 160 μL, 2, 6 DMP (2 mM), and 50 μL enzyme solution with a total reaction volume of 210 μL. The change in absorbance due to the oxidation of DMP was monitored at 469 nm for 20 min at an interval of 1 min. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of 2,6- DMP within a one-minute interval.

2.7. Xylanase and β-glucanase activity assays by DNS method

The selected strains were also examined for their ability to produce xylanase and β- glucanase following the method by Shrestha et al. [26]. This is performed by growing the selected high laccase positive strains from the study in the Czapek Dox medium with xylan, β-glucan, and lignin as the sole carbon source separately and checking the activity of cell-free supernatant after appropriate incubation. Xylanase activity was determined by incubating the crude enzyme with a suspension of 1% birchwood xylan in 0.1 M sodium acetate buffer solution at pH 4.0 and 30 °C for 5 min. The amount of xylose released during this time was then measured using the 3,5-dinitrosalicylic acid method (DNS). The reaction was halted by adding DNS, the samples were boiled for 10 min, cooled in water, and the optical density was measured at 540 nm. The xylanase activity was described as the amount of enzyme required to release 1 μmol of xylose per minute. The concentration of xylose was determined by creating a standard xylose curve using xylose standards with concentrations ranging from 100 to 800 μM.

Similarly, the β-glucanase activity was assessed by incubating the crude enzyme with 0.5% β-glucan at 30 °C for 5 min and then measuring the amount of reducing sugars released, using the 3,5-dinitrosalicylic acid method. Two controls were put up, lacking crude enzyme and substrate separately. One unit of β-glucanase activity was defined as the amount of enzyme required to release 1 μmol of glucose per minute under these conditions. The amount of glucose present was determined by comparing it to a standard curve which was created using glucose standards with concentrations ranging from 100 to 800 μM.

Furthermore, xylanase and β-glucanase activities in the selected strains were evaluated by cultivating them in CDB with lignin as the carbon source. Additionally, the strains were also grown in CDB media without any carbon source, and the activities of all three enzymes, xylanase, β-glucanase and laccase, were assessed.

2.8. Statistical methods

All the above experiments were performed in triplicate, and the average data were reported. Pearson's coefficient was used to evaluate correlation using GraphPad Prism 9.0.0. and p-value less than 0.05 was considered as the criterion for statistical significance.

3. Results and discussion

3.1. Establishing a high throughput screening process

Traditionally, lignin-degrading microorganisms have been selected based on qualitative methods such as direct screening using culture plates from environmental samples or by first enriching the environmental samples and then performing a plate-based screening [27]. The conventional screening process followed in previous studies is illustrated in the steps (left) shown in Fig. 1. To improve the reliability and efficiency of selected strains that highly express laccase enzymes using an HTP process, the methodology was changed by including a quantitative detection of laccase activity in the primary screening step as illustrated in the steps (right) shown in Fig. 1. The process applied in this study allows for the screening of large numbers of natural biomass samples (50 samples, 100 cultures), allowing quick identification of promising consortia. The HTP process was further applied in secondary screening by growing multiple colonies in a small volume that allowed quick isolation of high-activity strains. The identity of the selected strains was confirmed by sequencing, and the reliability of the screening process was further confirmed by growing up some of the selected strains in larger volumes and testing expressed enzyme activities.

3.2. Isolation and preliminary screening of laccase-producing bacterial and fungal strains

This study aimed to apply the HTP process to isolate potential ligninolytic microorganisms from the decayed wood samples collected near the Ottawa River in Ontario, Canada. A total of fifty wood samples were incubated by submerged fermentation and checked for total laccase activity quantitatively. Antimicrobials were added in the culturing medium separately for bacterial and fungal cultures, thus separating mixed microbial communities into each individual monoculture.

Out of 50 decomposed wood samples, eight bacterial cultures (A10, A12, A14, A16, A28, A32, A41, A50) and six fungal cultures (F10, F12, F14, F32, F41, F50) demonstrated high laccase activities ($\geq$ 50 U/L), with six samples overlapping in both groups and the other two only showed high activities in bacterial cultures and not in fungal cultures. Additionally, nine bacterial cultures (A2, A6, A18, A24, A30, A34, A36, A46, A48) and nine fungal cultures (F2, F6, F16, F28, F30, F34, F36, F46, F48) showed moderate laccase activity ($\geq$ 20 U/L), with seven overlapping samples (Fig. 2A, B). The remaining 19 bacterial cultures and 21 fungal cultures showed low activity ($<$ 20 U/L), and 14 wood samples showed no laccase activity under either culturing condition (Table 1). As a result, only a small percentage of the biomass samples - 16% for bacterial cultures and 12% for fungal cultures - underwent secondary screening, with the majority of samples (84% for bacterial cultures and 88% for fungal cultures) being excluded. Yet, a good number of both bacterial and fungal strainswith high laccase activities were isolated in secondary screening as presented in Section 3.3. Therefore, this methodology led to a significant reduction in labor and a considerable increase in the probability of identifying highly positive isolates.

3.3. Secondary screening for high laccase-producing strains

In the secondary screening, the wood samples from primary screening that exhibited high levels of total laccase activities were selected for isolating individual bacterial and fungal strains that express at least 20 U/L laccase activity. Subsequently, ten out of 82 bacterial isolates were chosen after isolating axenic cultures from primary screened wood samples. The potential bacterial strains selected were designated as AORB9, AORB10, AORB12, AORB28, AORB37, AORB46, AORB55, AORB25, AORB19, and AORB48 with laccase activity ranging from 22.31 to 65.77 U/L (Fig. 3A). Similarly, in the case of fungal strains, five out of 46 strains were selected that included AORF12,

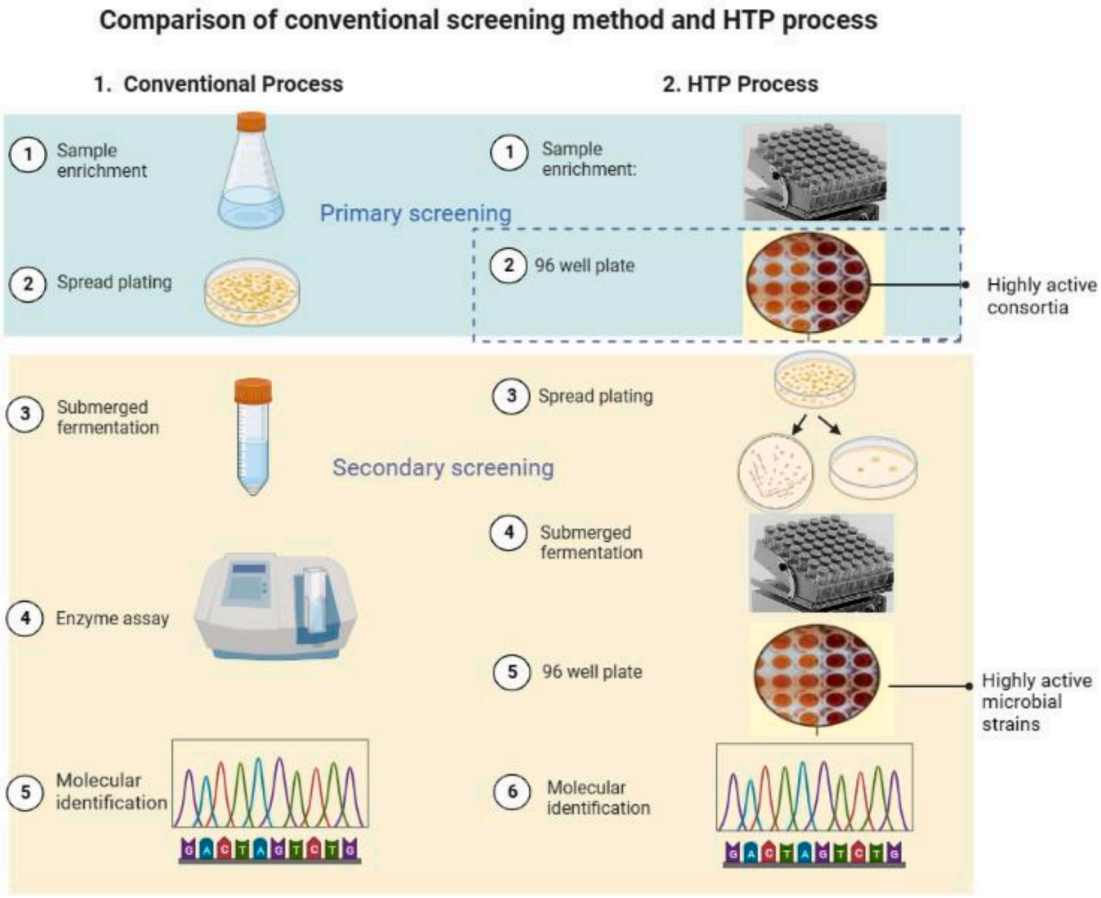

Fig. 1. Schematic of the screening workflow for ligninolytic microorganisms using the conventional method [6] and the HTP technique in this study.

AORF21, AORF39, AORF43, and AORF39, as they revealed laccase activities ranging from 33.19 to 41.51 U/L (Fig. 3B).

Total protein concentration in the microbial cultures was also examined in addition to the enzyme activity, and the majority of the strains ($P < 0.001$) with low enzyme activity exhibited low protein concentrations. It has been reported that, generally, high protein concentrations might be associated with higher enzyme activity [28]. However, it should be noted that protein concentration is not specific to a particular enzyme and can be affected by other factors, such as the growth conditions and the presence of other enzymes and proteins. For example, in the case of a few strains, high enzyme activity was noticed despite low protein concentration (AORB55, AORF21), and high protein concentrations with low enzyme activities were also observed (AORB73, AORF2) as depicted in blue dots in Fig. 3A, B. Assuming the majority of the expressed protein is composed of enzymes, then the laccase-specific activity of AORB55 would be much higher than AORB46, which showed similar activity but a much higher protein content. Future detailed identification and characterization of the specific enzymes would address this question.

3.4. Time course profile of laccase production by selected microbes

The selected microbial isolates were assayed to determine the time course of laccase production. To this end, time course studies were carried out for ten bacterial strains over a 120-h period with 24-h time intervals and five fungal strains over a 240-h period with 48-h time intervals. The results of the laccase activity of selected fungal and bacterial strains are shown in the heat map plot, Fig. 4A and B. The onset of laccase production occurred at different times and was not the same for all bacterial strains. Most tested bacterial strains showed significant laccase activities at 24 h and increased significantly at later hours, with AORB46 showing the highest activity at 96 h (69.42 U/L), followed by AORB55 (66.03 U/L). Further extended cultures after 96 h led to decreased activities in most strains except AORB10, AORB19, AORB28, and AORB37. In the case of fungal isolates, laccase production started at 96 h of incubation for AORF39 and AORF43, the other three stains started at 144 h, and enzyme activities kept increasing during the culturing period up to 240 h. Strain AORF21 (133.44 U/L) showed the highest activity, followed by AORF3 (121.10 U/L) and AORF12(119.35 U/L), respectively. AORF21 from the fungal isolates and AORB46 from the bacterial isolates demonstrated the highest laccase activity among each group.

3.5. Molecular identification of selected laccase-producing fungal strains

After the secondary screening, five potential fungal isolates were selected for genotypic identification based on internal transcribed spacer (ITS) gene sequencing analyses (Supplementary Figure. 2). BLAST search results of five ligninolytic fungal strains were distributed among four genera in 3 different taxa. This includes an ascomycete *Aspergillus sydowii* 49G11 (AORF21), two basidiomycetes: *Trametes versicolor* ANT213 QFB286 (AORF39) and *Coniferaporia weirii* JV0309 (AORF12) and two zygomycetes: *Mucor circenelloides* Pub005 (AORF39) and *Mucor* sp. CMRP 3219 (AORF43). Phylogenetic tree of fungal strains was constructed using the neighbor joining criteria of MEGA-X software (Supplementary Figure. 4 (a) to (e)).

Many species of the genus *Trametes* sp. including *T. versicolor* are known for their laccase production and its applications in denim bleaching [29]. Among the identified fungal strains (see Supplementary Table 2), the two Basidiomycetes - AORF39 and AORF12 are typical

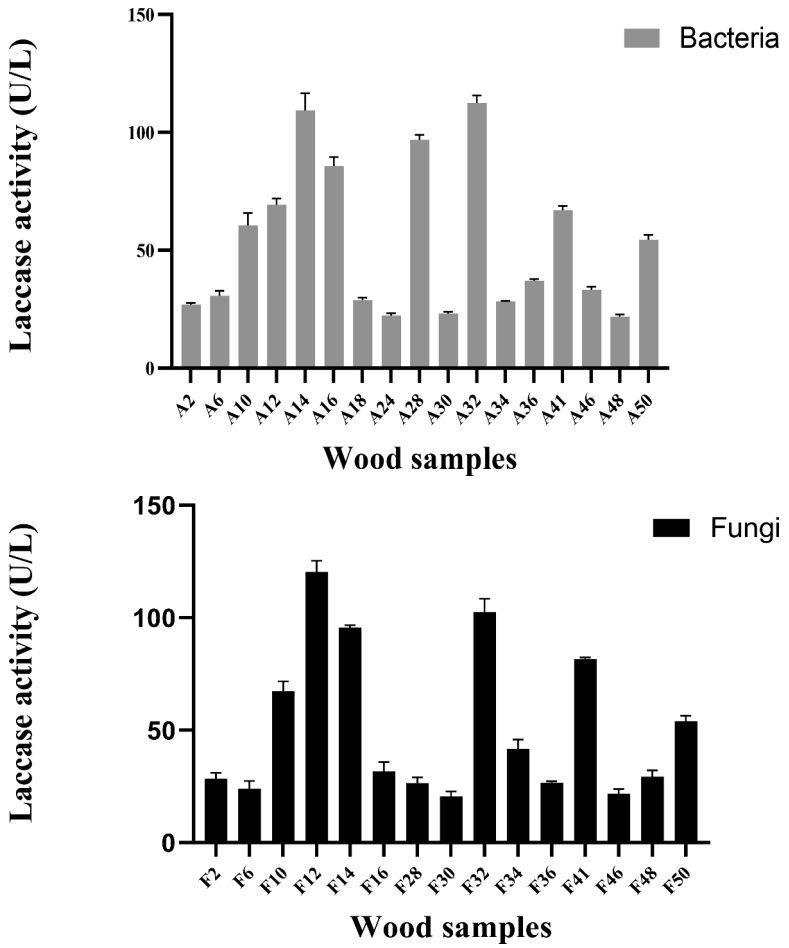

Fig. 2. Bar graph representing laccase activity of decomposed wood samples under bacterial (A) and fungal (B) culture conditions with high and moderate laccase activities. Wood samples with laccase activity ≥ 50 U/L were selected for secondary screening.

Table 1
Distribution of wood samples based on laccase activity in primary screening.

Laccase activity of wood samples in primary screening	Samples under bacterial growth conditions	Samples under fungal growth conditions
Low active wood samples (<20 U/L)	**19**	**21**
Moderately active wood sample (≥ 20 U/L)	**9**	**9**
High active wood samples (≥ 50 U/L)	**8**	**6**
Wood samples with no activity	**14**	**14**
Total wood samples	**50**	**50**

white rot fungi that have been reported as the most efficient lignin degraders in nature by the production of extracellular lignin-degrading enzymes [30]. Moreover, the white rot fungus *Coniferaporia weirri* (AORF12), formerly *Phellinus weirii*, is a destructive root pathogen of conifers, and its laccase production has been studied previously [31].

Ascomycetes also play an integral role in the recycling of lignin in nature. The strain *Aspergillus sydowii* NYKA 510 has been isolated from agricultural soil and has been reported as a powerful laccase producer, and has been successfully employed in a microbial fuel cell [32]. There are few studies on the involvement of lower fungi in laccase production, as Zygomycota do not utilize lignin and cellulose in general. However, few members of this phylum are confined to the outer layers of decomposing plant tissue and can break down lignin [33,34]. Moreover, there are very few studies on the role of lower fungi, zygomycetes, in lignin degradation. Among them, an indigenous litter-dwelling fungus *Mucor circinelloides* GL1 is reported as a promising laccase producer [35].

3.6. Molecular identification of selected laccase-producing bacterial strains

The BLAST search results of 16S rRNA gene sequence of ten chosen bacterial strains revealed gammaproteobacterial strains under three genera and one strain under phylum Firmicutes, class Bacilli as follows: *Serratia* (*Serratia* sp. CT197(AORB9), *Serratia proteamaculans* 336X (AORB19), *Serratia marcescens* AS09 (AORB28), *Enterobacter* (*Enterobacter* sp. XBGRY7(AORB10), *Enterobacter ludwigii* JUQ409 (AORB25), *Enterobacter hormaechei* EN 336X (AORB55), and *Raoutella* (*Raoultella ornitholytica* 4625 (AORB12), *Raoultella ornitholytica* Sch7(AORB37), *Raoutella ornitholytica* RT 1902(AORB9) and *Bacillus* sp. Ti1 (AORB48) (Supplementary Figure. 3). Phylogenetic tree of bacterial strains was constructed using the neighbor joining criteria of MEGA-X software (Supplementary Figure. 5 (a) to (j)).

All the ten potential laccase-producing bacterial species identified belonged to the phylum Gammaproteobacteria and class Bacilli (Supplementary Table 1). Gammaproteobacteria is a large and diverse category of bacteria with a wide range of phenotypes and metabolic capabilities. Their degradative traits are displayed in various nutrient-restrictive and xenobiotic habitats [36]. They have been isolated as one of the predominant bacterial phyla, which have been associated with lignin degradation from tropical forest soil [37,38]. Among the genus Serratia, *S. marcescens* isolated from a glacial site in India has been

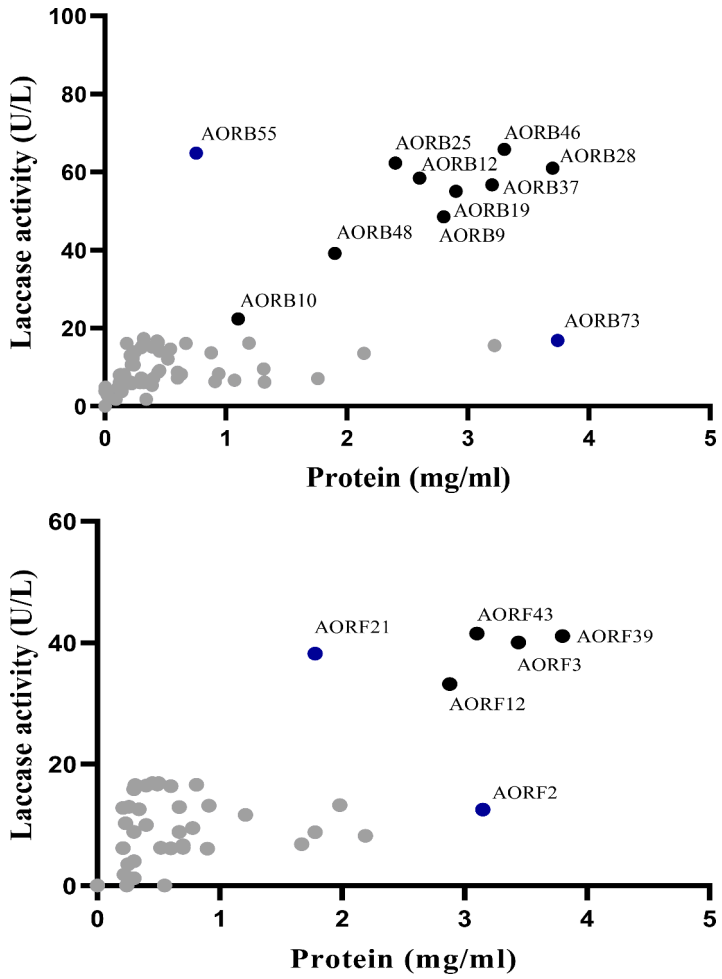

Fig. 3. (A) Scatter plot representing the total protein content and laccase activity on secondary screening of bacterial isolates. Bacterial strains which showed laccase activity ≥ 20 U/L were selected for molecular identification (B) Scatter plot representing the total protein content and laccase activity on secondary screening of fungal isolates. Fungal strains which showed laccase activity ≥ 30 U/L were selected for molecular identification. Black dots represent selected strains and grey dots represent non-selected strains. Blue dots showed representative strains with different enzyme activities and protein concentrations (color should be used).

shown to produce laccase, which can tolerate wide pH and temperature range [39]. The ability of *Serratia proteamaculans sp*. to secrete the laccase enzyme has been studied, and its cultural conditions were optimized [25]. In addition, *Enterobacter ludwigii*, isolated from decayed wood samples, has been shown to produce laccase and dye-decolorizing properties [40]. The bacterial species *Raoultella ornithinolytica* OKOH-1, isolated from sediment samples using culture enrichment, showed ligninolytic properties and held significant promise for use in various industrial applications, including the treatment of dye-contaminated wastewater management processes [41]. More so, there are several reports of the ability of bacterial strains under the genus *Bacillus* on lignin degradation [42,43].

Together, among the identified microbial strains, bacterial candidates AORB10, AORB12, AORB28 and fungal isolates AORF3 showed a sequence identity of 98%. Furthermore, fungal strains AORF43 and AORF39 along with bacterial strain AORB48 displayed sequence identities of 97%, 96% and 95%, respectively. Additionally, bacterial strains AORB55 and AORB37 showed sequence identities of 94% and 91%. Microbial isolates with sequence identity less than 99% compared to reference sequences may potentially represent novel species, and further studies are warranted for their classification. Moreover, a sequence identity below 95% identity is an indication of the possibility of a novel genus [44]. Thus, this methodology translates into an efficient and successful HTP screening process and isolation of unique lignin-degrading microorganisms. Although high throughput screening may expeditiously isolate an ensemble of microbes, they essentially might not unlock all entourage of microbes in an environmental sample due to the need for the preparation of varied culture media types based on nutritional requirements that promote growth to enhance their detection and isolation. Thus, the preparation of specific culture media takes precedence allowing for more efficient HTP screening of microbes, including unique isolates from the target samples. Furthermore, while biological lignin degradation is achieved by the combined action of multiple enzymes through diverse degradation pathways, this study was constrained by using laccase enzyme as the exclusive marker for evaluating the lignin-degrading traits of the isolates, consequently, may miss strains producing other enzymes instead of the detected one given the high throughput nature of the study.

3.7. Xylanase and β-glucanase activity profiles of selected microbial strains

The selected microbial strains were also assessed for their ability to produce other biomass hydrolyzing enzymes, such as *β*-glucanase and xylanase, on different culture media. The β-glucanase activity was determined in separate media using β-glucan and lignin as carbon sources. In the presence of β-glucan as the carbon source, *Aspergillus sydowii* 49G11 (AORF21) and *Trametes versicolor* ANT213QFB286 (AORF3) displayed β-glucanase activity of 3.13 U/ml and 2.92 U/ml, respectively. On the other hand, *Mucor circinellodes* Pub005 (AORF39)

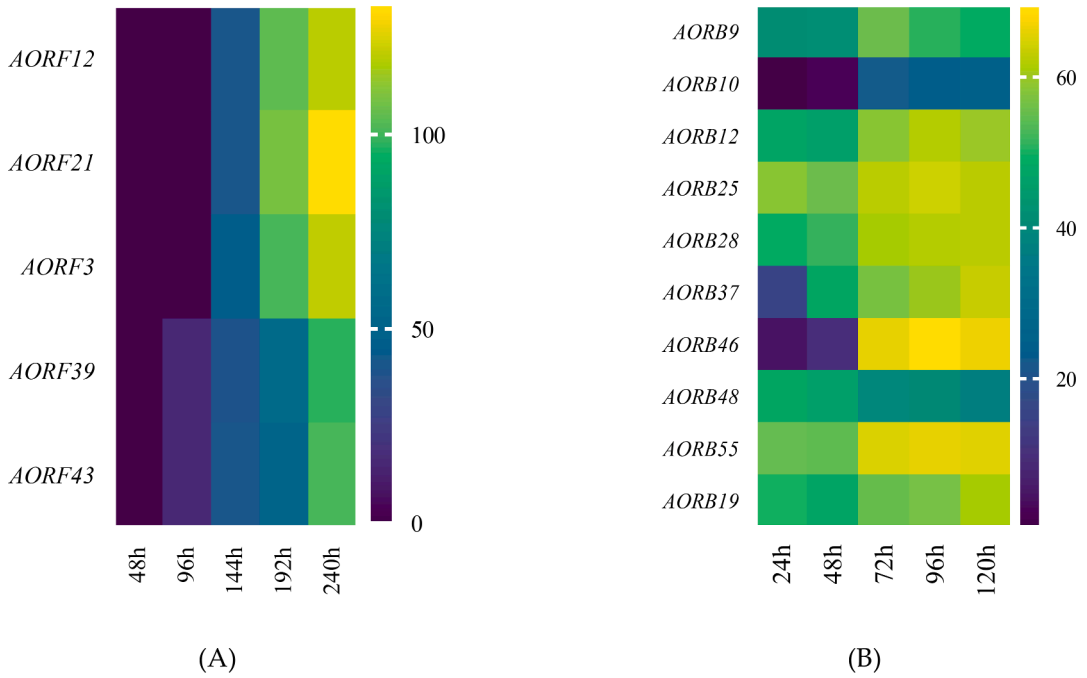

Fig. 4. Heatmap of the time course of laccase activity profiles of (A) fungal strains (B) bacterial strains. The purple-yellow color scheme was used to represent the laccase activity in the culture media of individual strains .

exhibited β-glucanase activity of 3.5 U/ml when lignin was used as the carbon source, while no enzyme activity was detected when β-glucan was the carbon source (Fig. 5).

Following this approach, Xylanase activity was measured in separate media using xylan and lignin as carbon sources. When xylan was the carbon source, *Aspergillus sydowii* 49G11 (AORF21) and *Trametes versicolor* ANT213 QFB286 (AORF3) exhibited xylanase activity of 4.09 U/ml and 3.22 U/ml, respectively. In contrast, *Mucor* sp. CMRP 3219 (AORF43) showed xylanase activity of 1.16 U/ml when lignin was the carbon source, with no enzyme activity detected when xylan was used. Furthermore, the bacterial strain *Enterobacter hormaechei* EN 314T (AORB55) demonstrated xylanase activity of 0.66 U/ml and 0.69 U/ml when xylan and lignin were used as the carbon sources, respectively (Fig. 6).

Aspergillus sydowii proved to be one of the best xylanase-producing strains isolated from shrimp shells [45]. *Trametes versicolor* has been extensively studied and is considered a highly promising strain due to its ability to produce numerous biodegrading enzyme [46]. *Mucor*

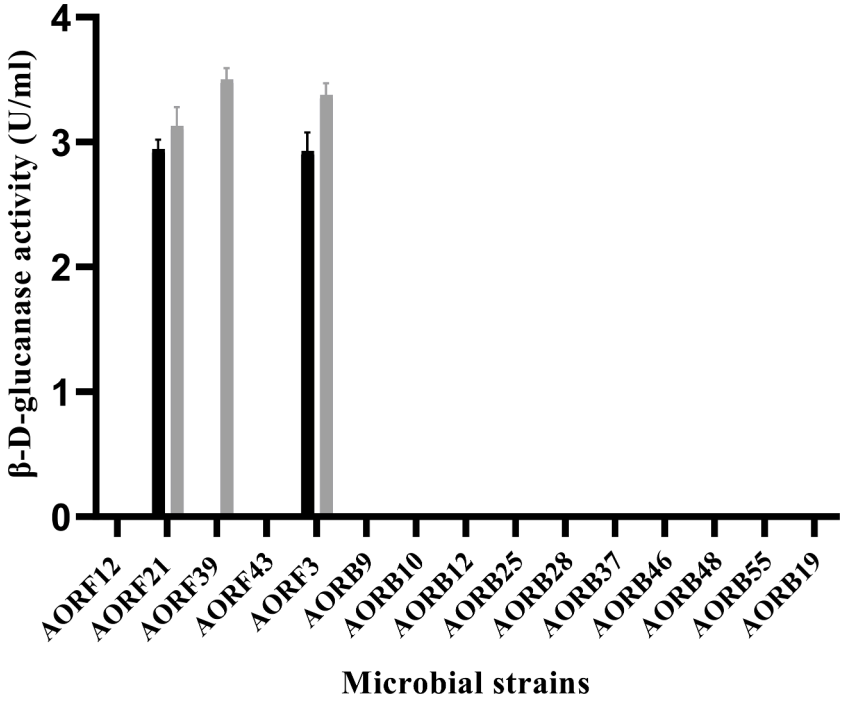

Fig. 5. Bar graph representing *β*-glucanase activity by screened microbial strains using β -glucan as carbon source (Black) and lignin (Grey) as carbon source. Error bars indicate the standard error of the mean of three replicates.

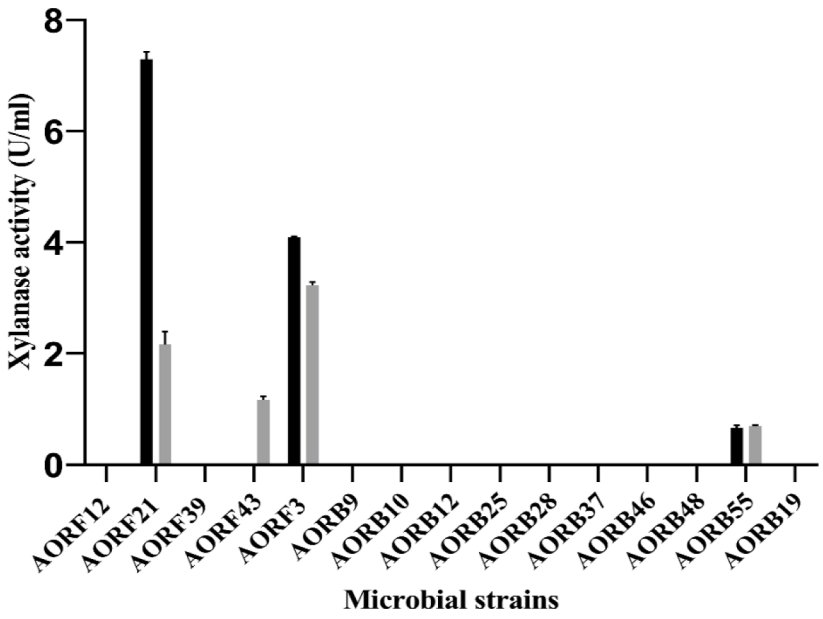

Fig. 6. Bar graph representing xylanase activity by screened microbial strains using xylan as carbon source (Black) and lignin (Grey) as carbon source. Error bars indicate the standard error of the mean of three replicates.

Circinellodes Pub005 and *Mucor* sp. CMRP 3219 exhibited β-glucanase and xylanase while using lignin as a carbon source. *Mucor circinelloides* is a fungus species that are dimorphic and extensively mentioned as a model producer of different enzymes that are useful for various industrial applications [47]. Additionally, the bacterial strain *Enterobacter* sp. exhibited xylanase activity while using both xylan and lignin separately. A heat and alkali-tolerant *Enterobacter* sp. was obtained from a sediment sample gathered from the Mandovi estuary located on the west coast of India and was determined to be an efficient xylanase-producing strain [48].

Biological lignin valorization is a complex multi-enzymatic process [49], including laccase, which is known to oxidize lignin employing molecular oxygen as the exclusive cofactor which sets laccase apart from other lignin-degrading enzymes - lignin peroxidases, manganese peroxidases and versatile peroxidase; that depend on H_2O_2 [50]. Laccase can cause the oxidative breakdown of phenolic units within lignin, resulting in the oxidation of the Cα position, cleavage of the Cα-Cβ bond, and cleavage of aryl-alkyl bonds. It can also attack the non-phenolic subunits of lignin through synthetic and natural mediators [51,52]. Several earlier studies have implicated laccase in the biodegradation of lignin, such as in *Sporotrichum pulverulentum*, *Coriolus versicolor* and *Trametes versicolor*, where laccase constitutes part of medley of enzymes causing lignin degradation in model compounds and treated lignin [53–55]. Additionally, *Pycnoporus cinnabarinus*, a fungal mutant strain devoid of laccase failed to secrete manganese peroxidase and lignin peroxidase, thus deteriorating its ability to metabolize synthetic lignin [56,57]. Furthermore, it was observed that a mutant strain of fungus, *Pleurotus*, lacking laccase enzyme activity exhibited inefficient degradation of lignin [50]. In line with these observations and findings, laccase was selected as a marker where microbial isolates are screened to identify the targeted lignin degrading microorganisms using a high throughput screening process.

In the primary screening, lignin was used as the sole carbon source that acts as a selective pressure to limit the growth of microorganisms that cannot use lignin as a single carbon source. This step focused the screening process towards strains that secreted high level ligninolytic enzymes needed to break down lignin polymer into smaller, more easily metabolizable compounds. Unexpectedly, a few, including both bacterial and fungal strains, also showed high-level cellulase and hemicellulase activities. Due to the fact that all enzymes are highly expressed under a single condition, these few strains may have significant valuable applications to be used for their combined or selective delignification ability in lignocellulosic biomass degradation.

Zhang et al. [58] obtained four lignin-degrading microbial consortia from 40 antique wooden samples in a tedious way. Each sample was grown up in a separate flask, and selections were based on subjective, visual color change. Even though the main fungal and bacterial compositions of the four consortia were identified by genomic sequencing, no specific strain was isolated. Similarly, from twelve samples of decayed tree trunks, stumps, and surrounding soil samples, Fang et al. [6] isolated a microbial consortium that demonstrated the ability to selectively break down lignin from tree trimmings where a chromogenic substrate was incorporated in the culture media that produced a color change when laccase is produced. The reported process is laborious and time-consuming as it takes months of culture and many generations of subcultures.

Xiong et al. [59] identified four bacterial strains from the soil, silage, and straw samples by first flask extraction of microorganism mixtures, followed by growth on ligninolytic selection agar media. Colonies that demonstrated aniline blue decolorization were chosen individually, and the strains were identified through 16S rDNA sequencing. There is no selection and screening of the starting biomass before single colony selection, and the total numbers of biomass samples tested were not reported. Recently, Elframawy et al. [60] screened 23 samples (14 soil samples and 9 old black liquor samples) to identify lignin degrading Actinobacteria (*Streptomyces* strains). Soil samples were first pretreated for 10 days, followed by the soil dilution plate technique (AGA plates with nystatin to reduce fungal growth), while black liquor samples were directly spread onto agar plates after dilution. Colonies were selected after 21 days of incubation, transferred to new AGA plates, and further cultured for 14 days to purify the isolates. The reported HTP approach not only allowed the quick identification of multiple fungal and bacterial strains but also some particularly interesting strains that can produce multiple, synergistic, and cohesive enzymes for efficient lignocellulose hydrolysis.

4. Conclusion

Innovative screening techniques allow the isolation of microbes and enzymes that have previously eluded detection and extend the

capability to discover novel enzymes. Essential to this endeavor, an HTP screening technique using a modified culture enrichment method for screening and selection of microbial strains from a large number of natural biomasses based on laccase activities was proposed and demonstrated in the study. This two-step strategy quickly identified promising consortia and isolated individual strains for efficient lignin and lignocellulose valorization, resulting in the identification of ten new bacterial and five new fungal strains with high lignin-degrading potential, including *Aspergillus sydowii* and *Raoutella ornitholytica* with the highest laccase activities. Moreover, the study evaluated the capability of the selected strains to produce other hydrolyzing enzymes, showing promising results for reducing enzyme costs in lignocellulosic biorefinery processes and discovering new enzymes with novel activities for the valorization of not only woody biomass but also biological waste, crops, and grains.

Funding

The manuscript corresponds to the National Research Council Communication #58388. The research project was supported by the NRC New Beginning Project Fund of the National Program Office Ideation Programs and the NRC Industrial Biotechnology Program awarded to TY. NA received support from the graduate scholarship of Lakehead University.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version.

Declaration of Competing Interest

The authors of the manuscript entitled "A high throughput screening process and quick isolation of novel lignin-degrading microbes from large number of natural biomasses" declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to extend their sincere appreciation to the Bioprocessing and Biocatalysis Team of ACRD Research Center, National Research Council; to Lakehead University, Thunder Bay, for their funding and financial support of this research, and Dr. Michel Gilbert, for critical review of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2023.e00809.

References

[1] H.P. Vu, L.N. Nguyen, M.T. Vu, M.A. Johir, R. Mclaughlan, L.D. Nghiem, A comprehensive review on the framework to valorise lignocellulosic biomass as biorefinery feedstocks, Sci. Total Environ. 743 (2020), https://doi.org/10.1016/j.scitotenv.2020.140630.

[2] T.C. Yang, J. Kumaran, S. Amartey, M. Maki, X. Li, F. Lu, W. Qin, Biofuels and bioproducts produced through microbial conversion of biomass, Bioenergy Res.: Adv. Appl. (2014) 71–93, https://doi.org/10.1016/B978-0-444-59561-4.00005-X.

[3] P. Adewale, A. Lang, F. Huang, D. Zhu, J. Sun, M. Ngadi, T.C. Yang, A novel *Bacillus ligniniphilus* catechol 2,3-dioxygenase shows unique substrate preference and metal requirement, Sci. Rep. 11 (2021), https://doi.org/10.1038/s41598-021-03144-8.

[4] S.S. Ali, A.E.-F. Abomohra, J. Sun, Effective bio-pretreatment of sawdust waste with a novel microbial consortium for enhanced biomethanation, Bioresour. Technol. 238 (2017) 425–432, https://doi.org/10.1016/j.biortech.2017.03.187.

[5] G. Jäger, J. Büchs, Biocatalytic conversion of lignocellulose to platform chemicals, Biotechnol. J. 7 (2012) 1122–1136, https://doi.org/10.1002/biot.201200033.

[6] X. Fang, Q. Li, Y. Lin, X. Lin, Y. Dai, Z. Guo, D. Pan, Screening of a microbial consortium for selective degradation of lignin from tree trimmings, Bioresour. Technol. 254 (2018) 247–255, https://doi.org/10.1016/j.biortech.2018.01.058.

[7] D. Tarasov, M. Leitch, P. Fatehi, Lignin-carbohydrate complexes: properties, applications, analyses, and methods of extraction: a review, Biotechnol. Biofuels 11 (2018) 269, https://doi.org/10.1186/s13068-018-1262-1.

[8] C. Chio, M. Sain, W. Qin, Lignin utilization: a review of lignin depolymerization from various aspects, Renew. Sustain. Energy Rev. 107 (2019) 232–249, https://doi.org/10.1016/j.rser.2019.03.008.

[9] R. Chandra, A. Raj, H.J. Purohit, A. Kapley, Characterisation and optimisation of three potential aerobic bacterial strains for kraft lignin degradation from pulp paper waste, Chemosphere 67 (2007) 839–846, https://doi.org/10.1016/j.chemosphere.2006.10.011.

[10] R. Chandra, S. Singh, M.M.K. Reddy, D.K. Patel, J. Purohit, H.A. Kapley, Isolation and characterization of bacterial strains *Paenibacillus* sp. and *Bacillus* sp. for kraft lignin decolorization from pulp paper mill waste, J. Gen. Appl. Microbiol. 54 (2008) 399–407, https://doi.org/10.2323/jgam.54.399.

[11] Z. Choolaei, R. Flick, A.N. Khusnutdinova, E.A. Edwards, A.F. Yakunin, Lignin-oxidizing activity of bacterial laccases characterized using soluble substrates and polymeric lignin, J. Biotechnol. 325 (2021) 128–137, https://doi.org/10.1016/j.jbiotec.2020.11.007.

[12] M. Maruthamuthu, D.J. Jiménez, P. Stevens, J.D.V. Elsas, A multi-substrate approach for functional metagenomics-based screening for (hemi)cellulases in two wheat straw-degrading microbial consortia unveils novel thermoalkaliphilic enzymes, BMC Genomics 17 (2016), https://doi.org/10.1186/s12864-016-2404-0.

[13] A. Raj, R. Chandra, M.M.K. Reddy, H.J. Purohit, A. Kapley, Biodegradation of kraft lignin by a newly isolated bacterial strain, *Aneurinibacillus aneurinilyticus* from the sludge of a pulp paper mill, World J. Microbiol. Biotechnol. 23 (2007) 793–799, https://doi.org/10.1007/s11274-006-9299-x.

[14] J.H. Tian, A.M. Pourcher, P. Peu, Isolation of bacterial strains able to metabolize lignin and lignin-related compounds, Lett. Appl. Microbiol. 63 (2016) 30–37, https://doi.org/10.1111/lam.12581.

[15] Z. Xu, L. Qin, M. Cai, W. Hua, M. Jin, Biodegradation of Kraft lignin by newly isolated *Klebsiella pneumoniae, pseudomonas putida*, and *ochrobactrum tritici* strains, Environ. Sci. Pollut. Res. 25 (14) (2018) 14171–14181, https://doi.org/10.1007/s11356-018-1633-y.

[16] H. Zhou, W. Guo, B. Xu, Z. Teng, D. Tao, Y. Lou, Y. Gao, Screening and identification of lignin-degrading bacteria in termite gut and the construction of lip-expressing recombinant *Lactococcus lactis*, Microb. Pathog. 112 (2017) 63–69, https://doi.org/10.1016/j.micpath.2017.09.047.

[17] J. Arnthong, C. Siamphan, C. Chuaseeharonnachai, N. Boonyuen, S. Suwannarangsee, Towards a miniaturized culture screening for cellulolytic fungi and their agricultural lignocellulosic degradation, J. Microbiol. Biotechnol. 30 (2020) 1670–1679, https://doi.org/10.4014/jmb.2007.07005.

[18] M. Bonnet, J.C. Lagier, D. Raoult, S. Khelaifia, Bacterial culture through selective and non- selective conditions: the evolution of culture media in clinical microbiology, New Microbes New Infect. 34 (2020), 100622, https://doi.org/10.1016/j.nmni.2019.100622.

[19] M. Neifar, H. Chouchane, M. Mahjoubi, A. Jaouani, A. Cherif, *Pseudomonas extremorientalis* BU118: a new salt-tolerant laccase-secreting bacterium with biotechnological potential in textile azo dye decolourization, Biotech 6 (2016) 107, https://doi.org/10.1007/s13205-016-0425-7.

[20] S. Sondhi, P. Sharma, S. Saini, N. Puri, N. Gupta, Purification and characterization of an extra- cellular, thermo-alkali-stable, metal tolerant laccase from *Bacillus tequilensis* SN4, PLoS ONE 9 (2014), https://doi.org/10.1371/journal.pone.0096951.

[21] T. Senthivelan, J. Kanagaraj, R.C. Panda, T. Narayani, Screening and production of a potential extracellular fungal laccase from *Penicillium chrysogenum*: media optimization by response surface methodology (RSM) and central composite rotatable design (CCRD), Biotechnol. Rep. (2019) 23, https://doi.org/10.1016/j.btre.2019.e00344.

[22] C. Zhu, G. Bao, S. Huang, Optimization of laccase production in the white-rot *fungus pleurotus ostreatus* (accc 52857) induced through yeast extract and copper, Biotechnol. Biotechnol. Equip. 30 (2016) 270–276, https://doi.org/10.1080/13102818.2015.1135081.

[23] G.K. Narde, A. Kapley, H.J. Purohit, Isolation and characterization of *Citrobacter* Strain HPC255 for broad-range substrate specificity for chlorophenols, Curr. Microbiol. 48 (2004), https://doi.org/10.1007/s00284-003-4230-2.

[24] I. Mukhtar, M. Arredondo-Santoyo, M.S. Vázquez-Garcidueñas, G. Vázquez-Marrufo, Isolation and molecular identification of laccase-producing saprophytic/phytopathogenic mushroom-forming fungi from various ecosystems in Michoacán State. Mexico, Acta Mycol. 54 (2019), https://doi.org/10.5586/am.1119.

[25] N.S. Ali, F. Huang, W. Qin, T.C. Yang, Identification and characterization of a new *Serratia proteamaculans* strain that naturally produces significant amount of extracellular laccase, Front. Microbiol. 13 (2022), https://doi.org/10.3389/fmicb.2022.878360.

[26] P. Shrestha, A.B. Ibáñez, S. Bauer, S.I. Glassman, T.M. Szaro, T.D. Bruns, J. W. Taylor, Fungi isolated from Miscanthus and sugarcane: biomass conversion, fungal enzymes, and hydrolysis of plant cell wall polymers, Biotechnol. Biofuels 8 (2015), https://doi.org/10.1186/s13068-015-0221-3.

[27] N.H. Abdul Rahman, N.A Abdul Rahman, S. Abd Aziz, M.A. Hassan, Production of ligninolytic enzymes by newly isolated bacteria from palm oil plantation soils, Bio. Res. 8 (2013), https://doi.org/10.15376/BIORES.8.4.6136-6150.
[28] M. Veerana, N.N. Yu, S.J. Bae, I. Kim, E.S. Kim, W. Ketya, H.Y. Lee, N.Y. Kim, G. Park, Enhancement of fungal enzyme production by radio-frequency electromagnetic fields, J. Fungi 8 (11) (2022) 1187, https://doi.org/10.3390/jof8111187.
[29] R. Bourbonnais, M.G. Paice, I.D. Reid, P. Lanthier, M. Yaguchi, Lignin oxidation by laccase isozymes from *Trametes versicolor* and role of the mediator2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) in kraft lignin depolymerization, Appl. Environ. Microbiol. (1995) 1876–1880, https://doi.org/10.1128/aem.61.5.1876-1880.1995.
[30] M.L. Niku-Paavola, T. Tamminen, B. Hording, L. Viikari, K. Poppius-Levlin, Reactivity of high and low molar mass lignin in the laccase catalysed oxidation, Prog. Biotechnol. (2002) 121–130, https://doi.org/10.1016/S0921-0423(02)80014-4.
[31] C.Y. Li, Phenoloxidase and peroxidase activities in zone lines of *Phellinus weirii*, Mycologia 73 (1981) 811, https://doi.org/10.1080/00275514.1981.12021412.
[32] Y.K. Abdallah, A.T. Estevez, D.E.D.M. Tantawy, A.M. Ibraheem, N.M. Khalil, Employing laccase- producing *Aspergillus sydowii* NYKA 510 as a cathodic biocatalyst in self-sufficient lighting microbial fuel cell, J. Microbiol. Biotechnol. 29 (2019) 1861–1872, https://doi.org/10.4014/jmb.1907.07031.
[33] C.T. Ingold, The Biology of Mucor and Its Allies. Studies in Biology No 88, Edward Arnold, London, UK, 1978.
[34] G. Janusz, A. Pawlik, J. Sulej, U. Świderska Burek, A. Jarosz-Wilkołazka, A. Paszczyński, Lignin degradation: microorganisms, enzymes involved, genomes analysis and evolution, FEMS Microbiol. Rev. 41 (2017) 941–962, https://doi.org/10.1093/femsre/fux049.
[35] P.A. Geethanjali, H.G. Gowtham, M. Jayashankar, Optimization of culture conditions for hyper- production of laccase from an indigenous litter dwelling fungus *Mucor circinelloides* GL1, Environ. Sustain. 3 (2020) 481–495, https://doi.org/10.1007/s42398-020-00137-7.
[36] J.O. Unuofin, A.I. Okoh, U.U. Nwodo, Recovery of laccase-producing gammaproteobacteria from wastewater, Biotechnol. Rep. 21 (2019), https://doi.org/10.1016/j.btre.2019.e00320.
[37] K.M. Deangelis, M. Allgaier, Y. Chavarria, J.L. Fortney, P. Hugenholtz, B. Simmons, K. Sublette, W.L. Silver, T.C. Hazen, Characterization of trapped lignin-degrading microbes in tropical forest soil, PLoS ONE 6 (2011) 6, https://doi.org/10.1371/journal.pone.0019306.
[38] G. Pold, J.M. Melillo, K.M. Deangelis, Two decades of warming increases diversity of a potentially lignolytic bacterial community, Front. Microbiol. 6 (2015), https://doi.org/10.3389/fmicb.2015.00480.
[39] G.S. Kaira, K. Dhakar, A. Pandey, A psychrotolerant strain of Serratia marcescens (MTCC 4822) produces laccase at wide temperature and ph range, AMB Express 5 (1) (2015), https://doi.org/10.1186/s13568-014-0092-1.
[40] O.A. Odeniyi, J.O. Unuofin, A.T.B.C. Wakil, S.A. Onilude, Production characteristics, activity patterns and biodecolourisation applications of thermostable laccases from *Corynebacterium Efficiens* and *Enterobacter Ludwigii*, J. Sci. Ind. Res. 76 (2017) 562–569.
[41] A.O. Falade, O.A.L. Eyisi, L.V. Mabinya, U.U. Nwodo, A.I. Okoh, Peroxidase production and ligninolytic potentials of fresh water bacteria *Raoultella ornithinolytica* and *Ensifer adhaerens*, Biotechnol. Rep. 16 (2017) 12–17, https://doi.org/10.1016/j.btre.2017.10.001.
[42] J. Mei, X. Shen, L. Gang, H. Xu, F. Wu, L. Sheng, A novel lignin degradation bacteria-*Bacillus amyloliquefaciens* SL-7 used to degrade straw lignin efficiently, Bioresour. Technol. 310 (2020), 123445, https://doi.org/10.1016/j.biortech.2020.123445.
[43] J. Yang, J. Zhao, J. Jiang, H. Xu, N. Zhang, J. Xie, M. Wei, Isolation and characterization of *Bacillus* sp. capable of degradating alkali lignin, Front. Energy Res. 9 (2021), https://doi.org/10.3389/fenrg.2021.807286.
[44] R. Schlaberg, K. Simmon, M. Fisher, A systematic approach for discovering novel, clinically relevant bacteria, Emerg. Infect. Dis. 18 (3) (2012) 422–430, https://doi.org/10.3201/eid1803.111481.
[45] S.C. Brandt, B. Ellinger, T.V. Nguyen, S. Harder, H. Schlüter, R.L. Hahnke, M. Rühl, W. Schäfer, M. Gand, *Aspergillus sydowii:* genome analysis and characterization of two heterologous expressed, non-redundant xylanases, Front. Microbiol. (2020).
[46] M. Tišma, P. Žnidaršič Plazl, G. Šelo, I. Tolj, M. Šperanda, A. Bucić-Kojić, M. Planinić, *Trametes versicolor* in lignocellulose-based bioeconomy: state of the art, challenges and opportunities, Bioresour. Technol. 330 (2021), https://doi.org/10.1016/j.biortech.2021.124997.
[47] C.E.R. Reis, H.B. Bento, A.K. Carvalho, A. Rajendran, B. Hu, H.F.D. Castro, Critical applications of *mucor circinelloides* within a biorefinery context, Crit. Rev. Biotechnol. 39 (2019) 555–570, https://doi.org/10.1080/07388551.2019.1592104.
[48] R. Khandeparkar, N.B. Bhosle, Purification and characterization of thermoalkalophilic xylanase isolated from the *Enterobacter* sp. MTCC 5112, Res. Microbiol. 157 (2006) 315–325, https://doi.org/10.1016/j.resmic.2005.12.001.
[49] S. Schoenherr, M. Ebrahimi, P. Czermak, Lignin degradation processes and the purification of valuable products, Lignin - Trends Appl. (2018), https://doi.org/10.5772/intechopen.71210.
[50] L. Munk, A.K. Sitarz, D.C. Kalyani, J.D. Mikkelsen, A.S. Meyer, Can laccases catalyze bond cleavage in lignin? Biotechnol. Adv. 33 (1) (2015) 13–24, https://doi.org/10.1016/j.biotechadv.2014.12.008.
[51] M.B. Agustin, D.M. Carvalho, M.H. Lahtinen, K. Hilden, T. Lundell, K.S. Mikkonen, Laccase as a tool in building advanced lignin-based materials, ChemSusChem 14 (21) (2021) 4615–4635, https://doi.org/10.1002/cssc.202101169.
[52] F.S. Archibald, R. Bourbonnais, L. Jurasek, M.G. Paice, I.D. Reid, Kraft pulp bleaching and delignification by trametes versicolor, J. Biotechnol. 53 (2–3) (1997) 215–236, https://doi.org/10.1016/s0168-1656(97)01675-1.
[53] P. Ander, K.E. Eriksson, The importance of phenol oxidase activity in lignin degradation by the white-rot fungus *Sporotrichum pulverulentum*, Arch. Microbiol. 109 (1–2) (1976) 1–8, https://doi.org/10.1007/bf00425105.
[54] R. Bourbonnais, M.G. Paice, Oxidation of non-phenolic substrates, FEBS Lett. 267 (1) (1990) 99–102, https://doi.org/10.1016/0014-5793(90)80298-w.
[55] S. Kawai, T. Umezawa, T. Higuchi, Degradation mechanisms of phenolic β-1 lignin substructure model compounds by laccase of *Coriolus versicolor*, Arch. Biochem. Biophys. 262 (1) (1988) 99–110, https://doi.org/10.1016/0003-9861(88)90172-5.
[56] H. Bermek, K. Li, K.E.-L. Eriksson, Laccase-less mutants of the white-rot fungus *pycnoporus cinnabarinus* cannot delignify Kraft pulp, J. Biotechnol. 66 (2–3) (1998) 117–124, https://doi.org/10.1016/s0168-1656(98)00091-1.
[57] C. Eggert, U. Temp, K.-E.L. Eriksson, Laccase is essential for lignin degradation by the white-rot fungus *Pycnoporus cinnabarinus*, FEBS Lett. 407 (1) (1997) 89–92, https://doi.org/10.1016/s0014-5793(97)00301-3.
[58] W. Zhang, X. Ren, Q. Lei, L. Wang, Screening and comparison of lignin degradation microbial consortia from wooden antiques, Molecules 26 (2021), https://doi.org/10.3390/molecules26102862.
[59] Y. Xiong, Y. Zhao, K. Ni, Y. Shi, Q. Xu, Characterization of ligninolytic bacteria and analysis of alkali-lignin biodegradation products, Polish J. Microbiol. 69 (2020) 339–347, https://doi.org/10.33073/pjm-2020-037.
[60] A. Elframawy, A. El-Hanafy, M. Sharamant, H. Ghozlan, Molecular identification of native Egyptian Actinobacteria: screening for lignin utilization and degradation of lignin model compounds, Biocatal. Agric. Biotechnol. 40 (2022), 102289, https://doi.org/10.1016/j.bcab.2022.102289.