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Qualitative and Quantitative Methods for Isolation and Characterization of Lignin-Modifying Enzymes Secreted by Microorganisms

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Abstract Lignin is a polyphenolic biopolymer synthesized by plants, for providing strength and rigidity to the plant cellular structure. It is closely associated with other plant polysaccharides in the cell wall such as cellulose and hemicellulose, constituting the most abundant biopolymer on the earth's surface. However, the complete utilization of it is being explored for the past few years. Various research groups around the world are trying to replace conventional fuels with the second-generation biofuels from lignocellulose. Several physical, chemical, and biological conversion methods have been developed for the separation and utilization of this biomass, as a result of which biological methods for lignocellulose conversion are considered to be cheap and environment-friendly. Microorganisms, especially fungi and bacteria, have been able to degrade the lignocellulose network and convert it to commercially important biofuels by secreting several intra- and extra-cellular enzymes. In the past few years, research has been conducted to isolate efficient lignin-degrading microorganisms, as separation of lignin from cellulosic biomass is considered as a major hurdle in biofuel and pulping industries. In this article, we extensively discuss different small- and large-scale methods developed for the isolation and characterization of lignin-degrading microorganisms. We have also comprehensively discussed about the qualitative and quantitative methods for the identification and characterization of the lignin-degrading and lignin-degrading auxiliary enzymes by comparing different methods based on their efficiency. This review can be used as a primer for understanding and selecting the most efficient method for isolation and characterization of lignin-degrading microorganisms and their enzymes.

Keywords Lignin · Cellulose · Biofuels · Plant biomass · Lignin oxidizing enzymes · Auxiliary enzymes · lignin-degrading microorganisms

Introduction

Forest biomass is one of the richest renewable resources on the earth's surface, which has a great potential for biobased products. These products can be produced by converting the major components of the plant cell wall such as cellulose, hemicellulose, pectin (carbohydrates), and lignin (polyphenolic) compounds. These components are usually present in inseparable complex networks, becoming highly abundant biopolymer. Lignin plays various roles in the plant development by providing strength, rigidity, and defense against microbial infections [1]. Compared to lignin, other plant biomass components such as cellulose and hemicellulose have been highly explored for the production of paper and biofuels. Various research groups around the world have developed several methods for the separation and utilization of lignin from the lignocellulose network using chemical, mechanical, physical, and biological methods. State of the art reviews on lignin valorization and pretreatment methods have been published in the last few years [2–4]. Compared to other methods, biological methods are considered to be ecofriendly and cheap, as these methods does not involve high energy for the removal of lignin from the lignocellulosic biomass [2]. However, commercially valuable materials such as carbon fiber,

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thermoplastic elastomers, engineering plastics, polymeric foams have been developed using chemical, physical, and mechanical methods [3, 4]. Microorganisms, especially fungi belonging to basidiomycetes phylum, have been able to degrade lignin along with cellulose and hemicellulose. These basidiomycetes fungi are divided into three classes (white, brown, and soft rot fungi), based on their wood-decaying patterns. Among these three classes of fungi, white rot fungi are highly studied for their ability to degrade lignin effectively, whereas brown and soft rot fungi degrade cellulose and hemicellulose components of the plant biomass readily but can slowly decompose lignin. At the same time, studies have also reported that some bacteria are able to successfully break down the lignin [5]. Bacteria such as *Streptomyces viridosporous* T7A and soil bacteria such as *Nocardia* sp., *Rhodococcus* sp., *Pseudomonas putida* mt-2, *Rhodococcus jostii* RHA1, and *Sphingomonas paucimobilis* SYK-6 were shown to degrade lignin considerably [5]. In the main, the bacteria showing lignin-degrading ability belong to actinomycetes, α-proteobacteria, and γ-proteobacteria. However, the ligninolytic ability of these bacteria is comparatively less than that of major white rot fungi such as *Phanerochaete chrysosporium* [5].

Two decades ago, the lignin-degrading ability of basidiomycetes fungi was credited for the generation of reactive oxygen species. Later, this was correlated by production of hydrogen peroxide in the lignolytic cultures of *Phanerochaete chrysosporium* [6]. Studies have reported the major lignin-degrading enzymes to be laccase, lignin peroxidase, manganese peroxidase and versatile peroxidases, and other H_2O_2-generating oxidases grouped under the designation "lignin-degrading auxiliary enzymes" [7] which have gained much attention due to their high ability to degrade lignin [6]. Nevertheless, studies have reported that these ligninolytic enzymes are significantly large in size and cannot penetrate healthy wood tissue, thus proving the role of reactive oxygen species in lignin decay [6]. Apart from these four major enzymes, there are other lignin-degrading enzymes which have been reported in recent years such as chloroperoxidase, superoxide dismutase, *Coprinus cinereus* peroxidases (CiP), novel peroxidase (NoP), and dye decolorizing peroxidases [8]. Thus, isolating and characterizing highly efficient lignin-degrading microorganisms will significantly impact present-day biofuel pulp industries and open new gateways in the production of commercially valuable biobased products. In this article, we have extensively reviewed a wide range of qualitative and quantitative methods for isolation and characterization of the lignin-degrading microorganisms and the intra- and extra-cellular enzymes secreted by these organisms. The methods described can be broadly classified into two main categories, low-cost preliminary methods and high-cost confirmatory methods (Fig. 1).

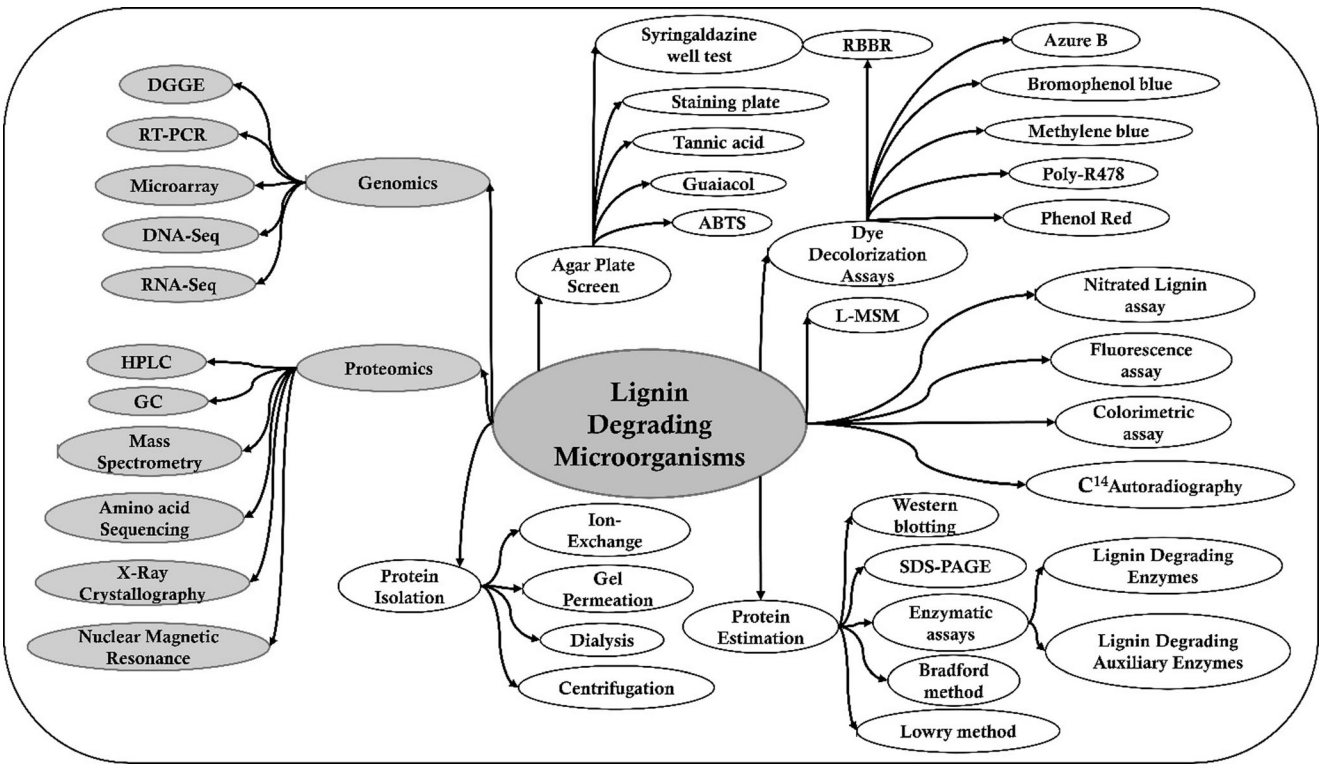

Fig. 1 A nested network of different methods used for the isolation and characterization of lignin-degrading microorganisms from decaying wood and soil samples. This image also differentiates methods to high throughput, costly methods (*grey colored ovals*), low throughput, less costly methods (*white colored ovals*)

Methods for Isolation and Characterization of Lignin-Degrading Microorganisms

Lignin Minimal Salt Medium (L-MSM) Potential lignin-degrading microorganisms can be isolated using enrichment culture technique [9]. Lignin enrichment media consist (g/l) of Na_2HPO_4 (2.4), K_2HPO_4 (2.0), NH_4NO_3 (0.1), $MgSO_4$ (0.01), $CaCl_2$ (0.01) and lignin or kraft lignin (1.0) [10–12]. Kraft lignin is obtained as a result of the kraft pulping process, which is performed to separate cellulose and remove a sufficient amount of lignin from the given plant biomass [13]. The above-mentioned components are thoroughly mixed and autoclaved then further transferred either to flasks (for liquid media) or to plates with agar (for solid media). According to Abhay et al. (2007), some bacterial strains cannot grow by using lignin or kraft lignin as a sole carbon source because of its high molecular weight and limited degrading abilities. In such situations, nutrients of the growth media can be optimized by providing 1.0 % glucose and 0.5 % peptone (w/v) in L-MSM broth or agar medium as subsidiary substrates [11]. L-MSM is an easy and low-cost method used for the preliminary isolations of lignin-degrading microorganisms. However, the microorganisms must be characterized for their lignin-degrading ability using advanced or specific methods, especially if the kraft lignin is used in the growth medium as it also contains some amounts of cellulose or hemicellulose which are easily degraded by microorganisms.

Dye Decolorization Method for Isolation Several studies have reported that lignin-degrading enzymes show great ability to degrade polychlorinated biphenyls (PCBs), polycyclic aromatics hydrocarbons (PAHs), chlorinated phenols, pesticides, and dyes [14–18]. Purified lignin-degrading enzymes were able to efficiently degrade or decolorize various dyes with different and complex chemical structures. Cripps et al. (1990) have reported that anaerobic bacteria convert the toxic azo dyes in to colorless carcinogenic compounds, however the same azo dyes not degraded under aerobic conditions [15]. Spadaro et al. (1992) have showed that *Phanerochaete chrysosporium* was able to efficiently decolorize and mineralize the toxic azo dyes to carbon dioxide [19]. Detoxification studies conducted by Heinfling et al. (1997) and Abadulla et al. (2000) have shown that fungal species such as *Trametes versicolor*, *Bjerkandera adusta* are able to significantly minimize the toxicity of azo and phthalocyanine dyes; in addition, immobilized laccase enzymes of *Trametes hirsuta* have reduced the toxicity of triphenylmethane, indigoid, azo, and anthraquinone dyes by 80 % respectively [17, 20]. Levin et al. (2004) have showed the primary isolation of lignolytic microbes by inoculated test strains on malt extract agar plates containing (12.7 g/l) malt extract, glucose (10 g/l), agar (20 g/l) and further supplemented with any one of six different dyes; Poly R-478 (0.02 %), Anthraquinone blue (0.02 %), Malachite Green (50 μM), azure B (50 μM), Congo Red and Xylidine Ponceau or Acid Red 26 [21]. Bandounas et al. (2011) have used the lignin-mimicking dyes such as azure B, Indigo Carmine, Malachite Green, Congo Red, Xylidine Ponceau, Methylene Blue, Toludine Blue O, and Remazol Brilliant Blue R (RBBR) for dye decolorization studies on both liquid and solid media [22]. According to Levin et al. 2004, dye decolorization tests should contain 0.02 % of anthroquinonic dyes whereas other dyes such as Malachite Green, Azure B, Congo Red and Xylidine Ponceau should be supplemented at 50 μM along with the growth medium [21]. Sample inoculum should consist of two agar plugs of 25 mm taken from a 5-day-old culture grown on MEA. In order to monitor the decolorization of the dyes two controls must be maintained; an uninoculated plate with the same dye concentrations acts as a control for abiotic decolorization, a control plate without any dye must be inoculated the same as the test plates. The test must be replicated three times; all the plates are incubated at 28 °C for 21 to 28 days. Dye decolorization is analyzed by measuring (a) the radial extension of the fungal mycelium, (b) average growth rates, calculated as cm/day, and (c) growth of fungal colonies and decolorized zones, calculated weekly. If the fungus is able to efficiently degrade the dye then a clear zone appears, and further, the samples are assessed quantitatively for enzyme activities [21]. The dye decolorization methods are cost-efficient and simple methods used for isolation of lignin-degrading bacteria or fungi, but these methods require further characterization using some advanced or specific methods which are discussed below.

Staining Lignin Agar Method Sundman and Nase (1971) wre the first to propose a simple and convenient method for estimating the lignin-degrading ability of the test strains. The composition of the assay medium involves 5 g ammonium tartarate, 1 g malt extract, 0.5 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2.{}_2H_2O$, 0.1 g NaCl, 0.01 g $FeCl_3$, kraft lignin (0.025 %) 1 mg thiamine, and 15 g agar in 1 liter of distilled water followed by adjusting the pH to 4.5; 1 ml of separately sterilized glucose (20 % w/v) is aseptically added to the final medium. The fungal growth medium is autoclaved and transferred to plates. Later, the plates are inoculated with the test strains and incubated for 5–10 days at 27 °C, followed by staining with freshly prepared 1 % w/v solutions of $FeCl_3$ and K_3 [Fe $(CN)_6$]. Plates show clear zones around the colonies indicating oxidized phenolic compounds; at the same time, phenols in undegraded lignin plates will stain blue green [23]. This method is used to show the degradation of phenolic compounds in lignin by the test strains; however, the degradation of non-phenolic lignin compounds is not shown by this procedure. Thus, this method does not prove the complete degradation of lignin by microorganisms [24, 25].

Tannic Acid Agar Method According to Pointing SB (1999), this method is a modification of the Bavendamm test (1928) [26] using tannic acid or gallic acid in the assay medium [23]. This assay is used to analyze the overall activity of polyphenoloxidase but is not specific to any of the lignin-modifying enzymes. The culture medium contains 1 ml of 20 % (w/v) glucose and 1 ml of 1 % (w/v) tannic acid which are sterilized separately and added aseptically to the autoclaved lignin basal medium (1.6 % w/v). The medium is aseptically transferred to petri plates, then inoculated with test strain and incubated at 27 °C in darkness. Appearance of a brown oxidization zone around the fungal colonies represents the overall polyphenoloxidase activity. However, natural brown colored pigment released by most of the fungi leads to further ambiguity in interpreting oxidization zones, which is considered to be the main disadvantage of this method [23, 26].

Autoradiography Using ^{14}C-ring Labeled Dehydrogenation Polymerizate Temp et al. (1998) has developed a small-scale method for the isolation of lignin-degrading microorganisms using ^{14}C-ring labeled dehydrogenation polymerizate [27]. However, for the first time Haider et al. (1975) have developed the most efficient method for determining the lignin-degrading ability of microorganisms, based on the measurement of $^{14}CO_2$ evolved from the ^{14}C-labelled cultures (^{14}C-ring-labeled dehydrogenation polymerizate) [28, 29]. However, the large scale analysis of lignin-degrading microorganisms using this method involves high cost and significant manpower, and at the same time it is also expensive with regard to the disposal of radioactive labelled lignin compounds. According to Temp et al. (1998), test fungal strains are primarily cultured on 2 % (w/v) malt extract agar for 10 days. Further 0.5 by 0.5 cm of agar blocks from the precultures are transferred to the wells of sterile tissue culture plates consisting of 3 ml of sterile basal liquid medium. These fungal cultures are incubated at 27 °C for 3 days and observed for the growth of mycelium prior to the addition of radiolabeled dehydrogenation polymerizate (DHP). On the 4th day, ^{14}C-DHP is dissolved in 5 μl of dimethylformamide and later added to each well. After 24 hours, sterile Whatman 3 mm filter paper is cut exactly to the dimensions of culture plates and soaked in sterile saturated barium hydroxide solution. The above soaked filter paper is further placed over the wells and closed firmly for the next 5 days at 30 °C. When $^{14}CO_2$ is evolved from the plates as a result of lignin degradation, it gets trapped in the filter papers as insoluble $BaCO_3$. Finally, the filter papers are removed and exposed to X-ray film for 5 days; development of dark color circles on the autoradiogram corresponds to the position well formed as a result of trapped $^{14}CO_2$. The intensity of the dark colored circles is directly proportional to the lignolytic capacity of the test fungal cultures. Finally, autoradiograms obtained from the above procedures are scanned and analyzed using any standard image analysis program. The whole experiment must be conducted with either two or three replicates; uninoculated culture plates with 3 ml of liquid basal medium (pH 4.6) along with radiolabeled DHP act as a control for the assay [27]. This method acts as a sensitive assay for identifying novel and efficient lignin-degrading microorganisms.

Colorimetric Assay Using Diazotized Derivative of Sulfonic Acid Rajan et al. (1992) developed a simple, rapid, and sensitive colorimetric assay for studying bacterial lignin degradation [30]. This spectrophotometric assay involves the reaction between diazotized sulfanilicacid and alkaline lignin solution [30]. The whole reaction contains three major steps: preparation of acid precipitable fraction of lignin, biodegradation of lignin samples by test strains, and reaction of diazotized sulfanilic acids with lignin. The detailed protocol and mechanism of the above steps is not discussed [30]. Though the method is simple and rapid, it has a disadvantage of being sensitive to non-lignin UV absorbing compounds which are generally observed in biological systems.

Spectrophotometric Assays Ahmad et al. (2010) proposed two spectrophotometric assays for the identification and screening of the lignin-degrading microorganisms [31]. This method can be used for rapid screening of the ligninolytic abilities of the microorganisms. According to Ahmad et al. (2010), lignin medium required for this assay was prepared based on the literature [32].

Fluorescence Assay for Screening Ligninolytic Microorganism In this assay, a fluorophore is attached to the lignin polymer, thus disruption or breakdown of the lignin polymer causes a change in the fluorophore's environment leading to a change in fluorescence. This method involves a fluorescent lignin medium prepared by mixing 5 mg of lignin in 0.5 ml of H_2O and 3 mg of (0.02 mM) K_2CO_3 thoroughly. The mixture thus obtained is further filtered using cotton wool; later, 100 μl of 0.2 mM fluorescein isothiocyanate (FITC) stock solution in water is added in aliquots for overnight stirring under a foil. Further, this solution is acidified, precipitated, and collected using centrifugation for 2 minutes at 10,000 rpm; the obtained lignin–fluorescein isothiocyanate (lignin-FITC) is used in the fluorescence assay. As the total volume of fluorescence assay constitutes 200 μl, assay can be performed in 96-well plates. The assay mixture consists of lignin-FITC stock solution containing 1 mg of lignin-FITC prepared in 5 mM Tris buffer with a pH 7.4 containing 50 mM NaCl and then diluted to 4.5 ml of Tris buffer. To each well, 160 μl of lignin-FITC stock solution is added along with 30 μl culture supernatant and 10 μl of 2 mM H_2O_2 solution. The change in fluorescence should be measured under a spectrophotometer at absorbance of λex 490 nm, λem 520 nm for every 1 minute for the first 10 minutes; after this, every

10 minutes for next 2 hours. The assay is conducted in duplicate for consistency, and assay mixture containing buffer solution replaced with either lignin or culture supernatant can act as control. The entire assay is performed by replacing H_2O_2 in the assay mixture with the buffer solution. The concentration dependence of the reaction can be inspected by performing the assay at different concentrations (10 μl, 30 μl, 50 μl) of culture supernatants. The fluorescence data vs time can be subtracted from the control data (without culture supernatant) using Microsoft Excel or other software. Generally, 10–30 % of error rate is typically observed between the duplicate assays [31].

Nitrated Lignin Assay This assay involves chemically nitrated lignin; disruption or degrading of lignin polymer releases nitrated phenolic compounds, thus resulting in increase in absorbance. As the name suggests this assay involves nitrated lignin for the qualitative determination of microorganisms. Ahmad et al. (2010) developed two methods for the preparation of nitrated lignin. In the first method, 5 mg of lignin and 3 mg of K_2CO_3 are mixed in 0.5 ml of water thoroughly and filtered to remove insoluble compounds. This is followed by the addition of 100 μl tetranitromethane to the above mixture during constant stirring under a foil at room temperature for 1 hour, and then centrifuged for 2 minutes at 10,000 rpm. Upon centrifugation, the whole solution is separated, the aqueous layer being removed to evaporate the remaining solution under vacuum, this method results in 0.6–1.3 mg nitrated lignin. For UV assay, a stock solution is prepared by mixing 1 mg of nitrated lignin in 100 ml of 750 mM Tris buffer at a pH 7.4 containing 50 mM NaCl. The second method involves 5 mg of lignin mixed in 1 ml of glacial acetic acid, and later the insoluble materials are filtered. Concentrated nitric acid 0.2 ml was added to the above reaction mixture at a constant stirring for 1 hour followed by adding 2 ml of water. This is followed by neutralization of the assay mixture to pH 7.4 using 1 M NaOH, resulting in a yellow-colored solution which is further diluted by 100-fold using 750 mM Tris buffer (pH 7.4) containing 50 mM NaCl which is used as stock solution for UV assay. The UV assay reaction mixture consists of a total mixture of 200 μl solution in each well of a 96-well plate, containing 30 μl of culture supernatant, 160 μl of nitrated lignin solution, and 10 μl of 40 mM H_2O_2 solution. The assay plates are constantly measured every 1 min time interval for 20 minutes using a spectrophotometer at an absorbance of 430 nm. In the assay mixture, either nitrated lignin or culture supernatant can be replaced by buffer solution, which acts as control to the assay reaction. The assay reaction can be performed in duplicates for consistency; however, about 10–30 % of error rate is typically observed between the replicates. Similar to that of the fluorescence assay, the concentration dependency of the reaction can be investigated using different concentrations (10 μl, 30 μl, 50 μl) of culture supernatants [31].

Denaturing Gradient Gel Electrophoresis (DGGE) DGGE is an undeniably significant approach for screening composite ecosystems on a large scale; it can easily analyze various environmental samples significantly in much less time. Recent studies have widely involved DGGE for assessing microbial population [33]. Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) has become a part of modern molecular microbial ecology [34]. Microbial ecology deals with studying intra-microbial, microbial, and environmental interactions; it involves long-term analysis involving various and numerous environmental samples. DGGE has gained higher importance in modern microbial ecology because most of the conventional methods such as cloning or hybridization are not always practical for microbial ecological investigations. These conventional techniques require an additional understanding of the microorganisms by developing adapted probes which can target specific microorganisms among diverse population, and also do not provide any information about environmental effects on microbial populations [34]. DGGE has the special advantage of visually profiling and monitoring changes occurring in different microbial communities which usually undergo different treatments. It is a rapid and efficient separation technique of similar length DNA sequences amplified by PCR, with chances of varying in single base pairs [34–36]. The DGGE method primarily involves the collection of DNA from the samples with multiple organisms; later, the collected DNA samples are amplified by PCR. Usually most conserved sequences such as 16S rDNA are considered for DGGE analysis. DGGE is performed at a constant temperature of 60 °C and under the influence of increasing concentrations of denaturing chemicals which forcefully separate DNA molecules. Separation of DNA molecules are based on electrical charge, molecular weight, and shape. The negatively charged DNA migrates through the pores of polyacrylamide gels, as it is attracted by the positively charged electrode. At a temperature of 60 °C and increasing concentrations of the denaturing agent's breakdown of the hydrogen bonds between the base pairs, the separation of DNA fragments occurs, which is said to be melted [34, 35]. This defines the melting regions [stretches of base pairs with similar melting temperatures example: base pairs containing A (adenine), T (Thymine) and those containing G (Guanine) and C (Cytosine) are melted apart]. Dissimilarity in DNA sequences within these regions will lead to different melting temperatures, thus causing differential migration of dissimilar sequences [35].

The above discussed isolation and characterization methods for lignin-degrading microorganisms were compared based on the cost effectiveness, time, and method efficiencies (Table 1).

Table 1 Comparison of different lignin-degrading microorganism isolation techniques

Isolation method	Time efficiency	Cost efficiency	Method efficiency	Reference
Colorimetric assay	+ +	$ $	+	[30]
Dye-decolorizing method	+ + +	$ $ $	+ +	[17, 20–22]
Lignin minimal salt medium (L-MSM)	+ + +	$ $	+ +	[10–12]
Staining agar plate	+ + +	$ $	+	[24, 25]
Tannic acid agar	+ + +	$	+ +	[23, 26]
C^{14} ring labeled dehydrogenation polymerizate	+ +	$ $ $ $	+ + +	[27]
Fluorescence assay	+ +	$ $ $ $	+ + +	[31]
Nitrated lignin assay	+ +	$ $ $	+ + +	[31]
Denaturation Gradient Gel Electrophoresis (DGGE)	+ +	$ $ $ $	+ + +	[33–36]

Time efficiency = 1– 5 days (+ + +), 5–10 days (+ +), 10 or more days (+); cost per gram = $ (1–10$), $ $ (10–20$), $ $ $ (20–100 $), $ $ $ $ (100$ or more); efficiency of method in characterizing lignolytic activity = methods which can prove lignolytic activity (+ + +), methods which require further confirmation (+ +), preliminary isolation methods (+)

Lignin-Oxidizing Enzymes (LO)

For the first time, Levasseur et al. (2008) classified the lignin-degrading enzymes into two major classes, as lignin-oxidizing (LO) and lignin-degrading auxiliary enzymes (LDA) [8]. Lignin-oxidizing enzymes include laccases (LO1), lignin peroxidases, manganese peroxidases, versatile peroxidases, chloroperoxidases (LO2), and cellobiose dehydrogenase (LO3). The LO2 class also includes the *Coprinus cinereus* peroxidases (CiP) and novel peroxidase (NoP) [8]. As the lignolytic activity of the microorganisms are highly dependent on the expression and activity of these enzymes, it is very important to assess the qualitative and quantitative properties to efficiently isolate lignin-degrading microorganisms (Fig. 2).

Qualitative Methods for Laccase

ABTS Agar Plate Assay ABTS (2-2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) is a well-known chemical compound used for studying the catalytic reactions of enzymes such as laccase and peroxidase enzyme systems. The assay medium consists of lignin minimal salt medium supplemented with 0.1 % w/v ABTS and 1.6 % w/v agar per 100 ml. The L-MSM with ABTS media is autoclaved, and later 1 ml of sterilized 20 % w/v aqueous glucose is added. The media is transferred and inoculated with test microbe aseptically, and incubated at 27 °C in dark conditions. The plates are constantly observed for 10 days, and development of green color must be noticed. Colorless ABTS-LMSM medium is turned to green, representing the presence of laccase and formation of ABTS-azine complex. The ABTS substrate can also be substituted with α-napthol (0.005 % w/v); upon laccase activity, a blue color is observed. This assay can also be used for interpreting the peroxidase enzyme systems, as peroxidases also oxidize ABTS, α-napthol in the presence of hydrogen peroxide [37–39].

Syringaldazine Well Test According to Harkin et al. (1974) and Niku Paavola et al. (1990), the syringaldazine test is an easy and simple method for determining the lignin-modifying enzymes secreted by a test fungus. Different reagents can be used for the well test; these reagents include ABTS, benzidine, guaiacol, gum guaiac, α-napthol, 1, 2, 3-trihydroxybenzene (pyrogallol), and syringaldazine. However, based on the chemical properties and availability, syringaldazine is the best choice for the well test. The culture medium consists of lignin basal medium supplemented with 1 ml (20 % w/v) of glucose, sterilized and aseptically added to the 100 ml medium. The medium is transferred aseptically to the plates followed by inoculating the test fungus, then incubated at 27°c in darkness for 5–10 days; the well test is performed by cutting 5 mm diameter wells in the agar growth medium. Laccase activity is determined by adding a few drops of 0.1 % w/v syringaldazine (in 95 % ethanol) to a well. Peroxidase activity can be determined by adding syringaldazine as the same concentration used for laccase activity along with a few drops of 0.5 % w/v aqueous H_2O_2 solution to the well. A few drops of 95 % ethanol are added to the wells, which will serve as control for both the tests. Appearance of purple color around each well in 30 minutes indicates laccase enzyme production. Production of peroxidase can be proved by a negative or less intense reaction for the laccase enzyme. Determining relative staining intensity of laccase and peroxidase reactions is not always easy, especially during rapid and intense color-developing reactions, posing some ambiguity in the result interpretation [37, 40].

Bromophenol Blue Plate Assay Bromophenol blue is a color marker with the chemical name (3'-3"-5'-5"-tetrabromophenolsulfonphthalein), also widely used as a

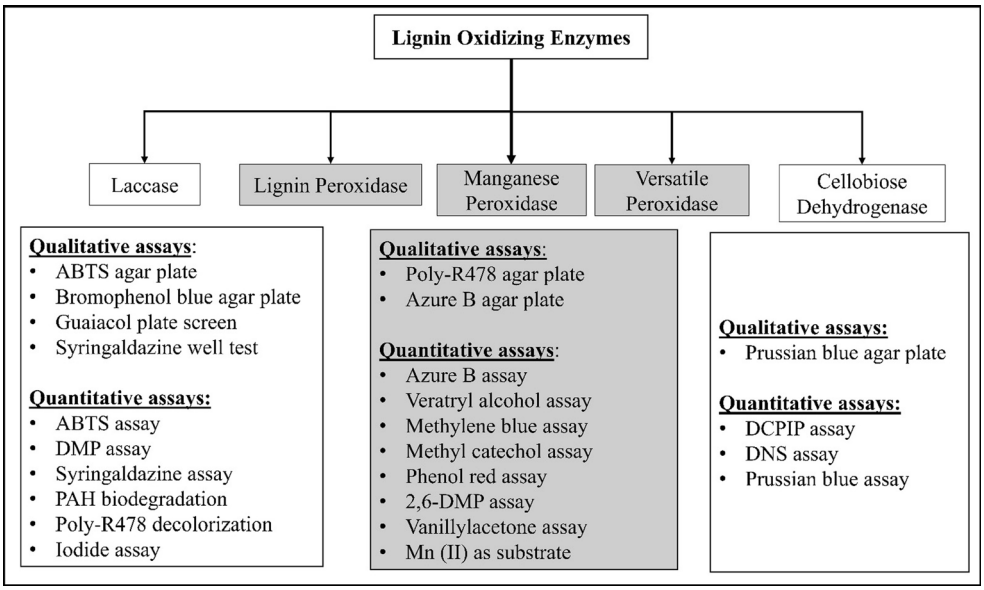

Fig. 2 Demonstrates various quantitative and qualitative assays of lignin-oxidizing enzymes

dye. Tekere et al. (2001) showed the dye-decolorizing abilities of lignin-degrading enzymes. Different dyes (Crystal Violet, Cresol Red and Blue Dextran), were used by Tekere et al.: however, Bromophenol Blue is one of the efficient dyes which can be used for quantitative detection of laccase enzyme. The dye-decolorization medium consists (w/v) of 2 % glucose, 3 % malt extract, 0.02 % dye, 0.1 % peptone (mycological), and 1 % agar. The plates are inoculated with test strains and later incubated at 27 °C to 30 °C based on the optimum temperature of the test strain. The degree of decolorization of the dye is visually examined by noticing clear halo zones around laccase-producing strains [25].

Guaiacol Agar Plate Assay Guaiacol is a naturally occurring yellow-colored aromatic compound derived from guaiacum. It is a product obtained upon pyrolysis of lignin present in wood smoke [41, 42]. Any particular assay media can be supplemented with 0.01 % guaiacol, Fatemeh et al. (2012) have used Nutrient agar supplemented with 0.5 mM guaiacol for the detection of laccase activity of *Bacillus subtilis WPI* [43]. The media containing guaiacol is autoclaved and transferred aseptically to the plates, followed by inoculation of test strain. The plates are incubated at 27 °C and 37 °C based on optimum temperature of test strain for 10 days; further plates are examined for the development of orange or brown color halo zones around laccase-producing microbial strains. Similarly, the drop screen procedure can be used for identifying laccase-producing bacteria, by preparing 12 μl of guaiacol to 1 ml of 100 % ethanol. A 2-day-old culture is considered, and later the above-prepared guaiacol solution is added drop by drop: the culture is kept aside for 4 hours and allowed to develop an orange or brown halo zone around laccase-producing fungal colonies [44, 45].

Quantitative Assays for Laccase

ABTS Assay ABTS is a non-phenolic dye which forms one of the most efficient substrates for laccase to study enzymatic reaction kinetics. ABTS is oxidized by the laccase enzyme to its more stable cation radical form. The final concentration of the cation radical in the reaction mixture is responsible for the development of intense blue–green color, which can be further correlated to laccase enzyme activity. The assay reaction mixture contains 0.5 mM of ABTS, 2.8 ml of 0.1 mM sodium acetate buffer with a pH 4.5, and 100 μl of culture supernatant. Test mixture is added and immediately incubated for 5 minutes, and later the absorbance is read at 420 nm using a spectrophotometer with a suitable blank. Laccase enzyme activity is further measured in units, one unit of laccase involved in the oxidization of 1 μ mol of ABTS substrate per minute [46]. Sodium azide (0.1 mM NaN_3) inhibits the whole reaction by lowering the concentration of ABTS cation radical by dilution, preventing the further formation of cation radical by completely inhibiting laccase enzyme [47].

DMP Assay 2,6-Dimethoxyphenol (2, 6-DMP) and other derivatives of it are plant phenolic compounds; these compounds are predominantly present in the thermal degradation of hardwood. DMP is one of the well-known substrates for the laccase enzyme, it has been widely used as a substrate for laccase enzyme to study the reaction kinetics, as it converts 2, 6-DMP to 3,5,3',5'-tetramethoxydiphenoquinone by resulting in a color change. The reaction mixture contains 10 mM DMP solution in 100 mM sodium acetate buffer with a pH 4.0 ($\varepsilon_{469} = 275001$ mM cm-1 referred to 2,6-DMP concentration). Whole assay reaction is carried out at room temperature (22–27 °C). The enzyme activity is demonstrated in units where one unit of enzyme activity is defined as the

amount of laccase oxidizing 1 μM of 2,6-DMP per minute [48–50].

Syngaldazine Assay According to Harkin and Obst (1973), syringaldazine (4-hydroxy-3,5 dimethoxy benzaldehyde azine) is an excellent substrate for easy and rapid detection of the laccase enzyme. Dilute syringaldazine solution in ethanol (mixture of alcohols or dimethyl sulfoxide) upon treatment with laccase enzyme changes its color from yellow to deep purple. The change in color is obtained from two-fold phenol dehydrogenation of syringaldazine and intramolecular pairing of the free radicals, resulting in highly colored tetramethoxy-azo-bis-methylene quinone complex. Unlike other substrates, color formation occurs when syringaldazine readily reacts with the laccase enzyme, and similarly color development is rapid at all pH conditions; at the same time, color fades rapidly outside the pH range of 3–7 [51]. The reaction mixture consists of syringaldazine (1.3×10^{-2} mM) in 0.1 mM phosphate buffer with pH 6.0 or 50 mM acetate buffer pH 4.5, and a suitable amount of enzyme is added to the reaction mixture. The reaction is continuously observed for the development of purple color (tetramethoxy-azo-bis-methylene quinone complex) at 30 °C by measuring its absorbance at 525 nm with a molecular extinction coefficient of 65,000 M^{-1} cm^{-1}. The oxidization of syringaldazine by laccase enzyme is determined in units where one unit of laccase oxidizes 1 μmol of syringaldazine per minute [52].

Mediator-Dependent Laccase Activity Assays

PAH Biodegradation Assay Several studies have reported concerning the efficiency of laccases in the oxidation of non-natural compounds such as polycyclic aromatic hydrocarbons (PAH's). Polycyclic aromatic hydrocarbons (PAH) are highly toxic organic pollutants exceedingly distributed in terrestrial and aquatic environments [53–57]. Laccase activity can be determined by oxidation of anthracene by reducing 9, 10-anthraquinone in the presence of sodium borohydride water-soluble solution. The method primarily involves the dilution of the laccase sample with acetate buffer at pH 5.0 or with enzyme diluent if required. Assay mixture containing 50 μl of laccase sample and 50 μl of PAH reaction solution is transferred to the 96-well plate. Anthracene and mediator compounds can act as controls for checking the PAH autoxidation (it can contain 50 μl of PAH but the laccase should be replaced). Contents of the reaction mixture must be thoroughly mixed with either single or multi-channel pipette; the wells of the 96-well plate are covered using a sealing film and incubated in darkness at room temperature for 24 hours. After 24 hours, the oxidation reaction of laccase is stopped by adding 100 μl of 100 % ethanol and 20 μl of sodium borohydride water-soluble solution (SWS) followed by thorough mixing of the reaction contents. The plate is sealed again and incubated at room temperature for 15 minutes; then the absorbance of the reaction mixture is taken using a spectrophotometer at 419 nm. One unit of PAH-activity can be measured by the amount of enzyme which produces 1 μmol of 9,10-anthroquinone per minute under the described conditions [58].

Decolorization Assay Using Poly-R478 Poly-R478 is a well-known lignin model compound related to the class of PAHs; thus, both types of compound can be degraded by the laccase mediator system. Polymeric dye decolorization was developed based on its correlation with PAH biodegradation. Similarly to the PAH biodegradation method, initially laccase samples are diluted using acetate buffer (pH 5.0) or with enzyme diluent if required. The reaction mixture consists of 50 μl of laccase sample and 150 μl of Poly-R478, with controls having the same amount of Poly-R478 as the reaction mixture but without laccase. The contents of the reaction mixture are thoroughly mixed and the absorbance of the reaction is constantly monitored under spectrophotometer at 520 nm. Later, the plates are sealed with sealing film and incubated for 15 minutes to 6 hours at room temperature, based on the amount of the enzyme added. Laccase enzyme activity can be measured based on its ability to decolorize Poly-R478; one unit of decolorization activity of laccase is equal to 1 μmol of Poly-R478 per minute under the described conditions [58–60].

Iodide Assay This is a mediator-dependent laccase assay which catalyzes the oxidation of iodide to iodine [61]. At the same time, in situ generation of iodine from iodide using laccase is an advantageous reaction for industrial and medical sterilization purposes when compared to peroxidase-based systems, as it uses dioxygen instead of peroxide. The method commences by diluting the laccase samples with enzyme diluent. The reaction mixture contains 20 μl of laccase sample and 180 μl of iodide assay solution. The total reaction mixture is mixed thoroughly, and the absorbance is recorded at 353 nm using a plate reader. Then the plate is sealed using a sealing tape and incubated at room temperature in dark conditions for 1–6 hours. After incubation, the sealing film is removed and absorbance is recorded again at 353 nm, for calculating the relative activities based on the difference between primary absorption followed by dividing by the incubation time (in minutes). The laccase enzyme activity is measured based on the oxidization of 1 μmol iodide/min at its corresponding conditions [58, 61].

All the above-discussed qualitative and quantitative methods for the characterization of laccase enzymes were compared with respect to their cost, time and method efficiencies (Table 2).

Table 2 Comparison of different qualitative and quantitative methods for the identification and characterization of laccase enzyme

Isolation method	Method type	Time efficiency	Cost efficiency	Method efficiency	Reference
ABTS plate assay	Qualitative	+ +	$ $ $ $	+ + +	[37–39]
Syringaldazine assay	Qualitative	+ +	$ $ $ $	+ +	[37, 40]
Bromophenol blue	Qualitative	+ +	$	+	[25]
Guaiacol plate assay	Qualitative	+ +	$	+ +	[44, 45]
ABTS enzyme assay	Quantitative	+ + +	$ $ $ $	+ + +	[47, 62]
Syringaldazine assay	Quantitative	+ + +	$ $ $ $	+ + +	[51, 52, 63]
DMP assay	Quantitative	+ + +	$	+ + +	[49, 50]
PAH biodegradation	Quantitative	+ + +	$ $ $	+ +	[58]
Poly-R478 decolorization	Quantitative	+ + +	$ $ $ $	+ +	[58–60]
Iodide assay	Quantitative	+ + +	$ $	+ +	[58, 61]

Time efficiency = 1– 2 days (+ + +), 3–10 days (+ +), 10 or more days (+); cost per gram = $ (1–10$), $ $ (10–20$), $ $ $ (20–100 $), $ $ $ $ (100$ or more); efficiency of method in characterizing lignolytic activity = methods which can prove lignolytic activity (+ + +), methods which require further confirmation (+ +), preliminary isolation methods (+)

Qualitative Assays for Peroxidases

Poly-R478 Agar Plate Studies have shown that fungal ability in decolorization of Poly-R478 (a polymeric dye) is correlated with secretion of several lignin-degrading peroxidases or poly phenoloxidases [64–66]. This screening method involves the L-MSM or any microbial growth medium supplemented with (w/v) of 0.02 % of Poly-R478 with 1.6 % of agar and 1 ml of 20 % (w/v) of glucose (aqueous) for 100 ml of screening medium. Later the medium is aseptically transferred to the plates and inoculated with test strain and incubated at 27 °C (in darkness). Screening plates are examined daily for 10 days, and production of lignin-degrading peroxidases can be inferred by observing a clear halo zone around the test colony in the violet-colored medium. Decolorization of Poly-R478 is one of the most significant and convenient qualitative methods for determination of lignin-degrading peroxidases. Compared to other assays, peroxidases can be easily estimated without the addition of hydrogen peroxide to the medium. However, there is some ambiguity when this assay is used for laccase estimation, as laccase secreted by several tropical marine fungi fails to decolorize Poly-R478 [66]; at the same time, laccases secreted by several terrestrial basidiomycetes are able to decolorize Poly-R478 [65]. The decolorization ability of the test fungus can also be estimated by using broth cultures and estimating the degree of decolorization by spectrophotometer at an absorbance ratio of 520/350 nm [25].

Azure B Agar Plate Assay Several fungi which secrete lignin peroxidase and manganese peroxidase also have the ability to decolorize or degrade Azure B dye. However, laccase enzyme cannot decolorize Azure B; thus, it can be used as a qualitative test for ligninolytic peroxidase production. The assay medium contains lignin minimal salt medium (L-MSM) or any microbial growth medium supplemented with 0.01 % w/v Azure B and later 1 ml of 20 % w/v aqueous glucose solution per 100 ml of assay medium. The medium is autoclaved and aseptically transferred to the plates, and inoculated with test fungus and incubated at 27 °C in dark conditions. The test plates are examined regularly and monitored for the appearance of clear halo zones around fungal colonies. Clearance of blue color in the assay plates confirms the production of lignin and manganese peroxidase. One of the advantages of using the Azure B clearance test is that it doesn't require the addition of hydrogen peroxide to the assay medium [67].

Methylene Blue Assay Magalhaes et al. (1996) showed that methylene blue can be used as the simple and rapid visual inspection assay for lignin peroxidase enzyme activity in the culture supernatant. The assay mixtures contain 2.2 ml of culture supernatant (without dialysis), 0.1 ml of 1 mM methylene blue, 0.3 ml of 0.5 M sodium tartarate buffer with a (pH 4.0), and 0.1 ml of 4.5 mM H_2O_2. Oxidative reaction of the lignin peroxidase will start after the addition of H_2O_2. The test mixtures should be closely examined to observe a change in color from greenish blue to purple blue. The time taken for color development can be correlated to lignin peroxidase activity of the test strain. The color that develops in the presence of lignin peroxidase is compared to a blank assay; twice-distilled water is used to replace the supernatant [68].

Methyl Catechol Assay Brown et al. (1993) have reported methyl catechol assay for determining the production of manganese peroxidase enzyme qualitatively in the culture supernatants. The assay mixture consists of 0.4 mM methyl catechol, 0.2 mM $MnSO_4$, and 50 mM sodium succinate buffer solution with a pH of 4.5 and 0.1 mM H_2O_2. The oxidative reaction is initiated by the addition of H_2O_2 to the reaction mixture. The text mixture

should be closely observed for the color change from pale yellow–brown to orange–brown or yellowish–brown. The speed of color change is directly proportional to MnP activity of the test culture. Inactivated culture supernatants or double-distilled water can be used for blank assay [69].

Quantitative Assays for Lignin Peroxidases

Veratryl Alcohol Oxidation Veratryl alcohol is a well-known substrate for the lignin peroxidase (LiP) and is frequently used for quantification of LiP. The method is based on the oxidation of veratryl alcohol (3, 5-dimethoxybenzyl alcohol) to veratraldehyde. The reaction mixture contains 1 ml of 125 mM sodium tartarate buffer (pH 3.0), 500 μl 10 mM veratryl alcohol, 500 μl of 2 mM H_2O_2 solution, and 500 μl of culture filtrate of test strain. The reaction can be performed in a 96-well plate by thoroughly mixing the contents; reaction is initiated by adding H_2O_2. The change in absorbance is continuously monitored using a spectrophotometer at 310 nm. Enzyme activity can be evaluated as one unit of enzyme activity for the oxidation of 1 μmol of veratraldehyde (oxidized product of veratryl alcohol) produced per minute per ml of culture filtrate of test strain [70].

Azure B Assay Archibald et al. (1992) have developed enzymatic assay using Azure B for the quantification of lignin peroxidase [67]. Several fungi with ability to secrete lignin peroxidase also have the ability to decolorize Azure B dye; thus, it is also used as a qualitative test for the primary isolation of lignin-degrading microorganisms [23]. The reaction mixture for Azure B assay contains 1 ml of 50 mM sodium tartarate (pH 4.5 or 2.5), 0.1 mM hydrogen peroxide, and 32 μM Azure B. Similarly to the VA assay, reaction is initiated by adding H_2O_2, resulting in a change in absorbance or decolorization of the substrate Azure B, which is monitored at 651 nm using a plate reader at an interval of 1 minute for a period of 30 minutes [67, 71].

Manganese Peroxidase Assays According to Paszczyński et al. (1988), manganese peroxidase can be quantified using a variety of aromatic compounds, especially those compounds which are used for the assays of common peroxidases such as horseradish peroxidase. However, the reaction mixture should contain Mn (II) ions — potential substrates used for MnP activity and their respective wavelengths are: (a) TMPD (N,N,N,N-Tetramethyl-1,4-phenylenediamine (2HCl)) (610 nm), (b) vanillylacetone (336 nm), (c) 2,6-dimethoxyphenol (568 nm), (d) syringicacid (260 nm), (e) guaiacol (465 nm), (f) curcumin (430 nm), (g) syringaldazine (525 nm), (h) coniferyl alcohol (263 nm), and (i) *o*-dianisidine (−2HCl) (460 nm) [72]. The reaction mixtures using the above substrates generally consist of 0.5 M sodium tartarate buffer (pH 5.0), 1 mM substrate dissolved in water (for water-soluble compounds), or 50 % aqueous N',N-dimethylformamide (for water-insoluble compounds), 1 mM $MnSO_4$, 1 mM H_2O_2, and enzyme solution or culture filtrate containing about 0.2 U/ml of enzyme. The reaction is initiated by adding hydrogen peroxide; reaction is monitored using spectrophotometer, and the wavelength is set based on the substrate used [72].

Phenol Red Assay Phenol red assay is one of the widely used assays for determination of manganese peroxidase (MnP). The reaction mixture consists of 25 mM lactate, 0.1 mM $MnSO_4$, 1 mg of bovine serum albumin, 0.1 mg of phenol red per ml, and 0.5 ml of culture filtrate in 20 mM sodium succinate buffer with a pH of 4.5, making a total volume of 1 ml. The reaction is initiated by adding hydrogen peroxide to the reaction mixture by making the final concentration to 0.1 mM. The enzymatic reaction is stopped after one minute by adding 50 μl of 10 % NaOH; the whole reaction is monitored at an absorbance of 610 nm. The reaction mixture without Mn^{2+} (without adding $MnSO_4$) acts as a control for the phenol red oxidation assay. Manganese peroxidase enzyme activity is calculated by subtracting the phenol red oxidizing activity values without Mn^{2+} (control) from the phenol red oxidizing activity values with Mn^{2+} (test), which is expressed as A_{610} per minute per milliliter [73, 74].

Vanillylacetone Assay Vanillylacetone (4-(4-hydroxy-3-methoxyphenyl)-2-butanone) is also called zingerone, and it is structurally similar to vanillin or eugenol, routinely used for manganese peroxidase assay. Manganese peroxidase can be assessed using vanillylacetone as a substrate [75]. The reaction mixture consists of 0.1 mM vanillylacetone (substrate), 0.1 mM $MnSO_4$, 0.05 mM H_2O_2, 100 mM sodium tartarate with pH of 5.0, and enzyme or culture filtrate is added to make the total volume 1.0 ml. The contents of the reaction mixture are mixed thoroughly, followed by addition of hydrogen peroxide to initiate the reaction. Disappearance of vanillylacetone is monitored using spectrophotometer at absorbance of 336 nm($\varepsilon_{336} = 18{,}300$); one unit of manganese peroxidase activity is defined as 1 μmol of vanillylacetone oxidized per minute [75].

2,6-Dimethoxyphenol Assay Manganese peroxidase enzyme activity can be assessed directly by observing the formation of Mn^{3+} tartarate complex during oxidation of 0.1 mM $MnSo_4$ with a molar extinction coefficient of 6500 M^{-1} .cm^{-1} (Mn^{2+}-dependent peroxidase activity). Manganese peroxidase is also indirectly quantified based on its oxidization of 2,6-dimethoxyphenol to 3, 3', 5, 5'-tetramethoxy-p, p'-diphenoquinone (Mn^{2+}-independent peroxidase activity). Reaction mixture consists of 1 mM 2,6-DMP, 0.1 mM hydrogen peroxide, 1 mM $MnSO_4$, and 100 mM sodium tartarate (pH 4.5). This assay is mainly used to determine the manganese-independent peroxidase activity; manganese

peroxidase activity is corrected for manganese-independent peroxidase activity by subtracting the activity obtained at pH 4.5 in the absence of Mn^{2+} ($MnSO_4$). The oxidation of 2,6-DMP is monitored using spectrophotometer at absorbance of 469 nm; one unit of manganese peroxidase activity is defined as 1 μmol of DMP oxidized per minute [76].

Mn (II) as Substrate According to Paszczyński et al. (1988), oxidation of Mn (II) to Mn (III) is the most convenient assay for determining the activity of manganese peroxidase [72]. Usually, purified preparations of MnP are best suited for this assay, as the contaminating metals such as iron and copper inhibit the MnP activity. Reaction mixture consists of 0.1 M sodium tartarate (pH 5.0), 0.1 mM hydrogen peroxide, 0.1 mM $MnSO_4$, and the culture filtrate or enzyme. The resulting product, Mn (III), forms a stable complex transiently with tartaric acid, which can be monitored at an absorbance of 238 nm using spectrophotometer during first 5 to 30 seconds of the reaction. Similarly to all other assays, reaction is initiated by adding hydrogen peroxide; one unit of MnP oxidizes 1 μmol of Mn (II) per minute [72].

Table 3 compares the cost, time, and method efficiencies of the above-discussed quantitative and qualitative techniques for the characterization of ligninolytic peroxidases (Table 3).

Qualitative Assay for Cellobiose Dehydrogenase

Prussian Blue Agar Plate Screen Vasilchenko et al. (2012) developed a high-throughput method for the screening of cellobiose dehydrogenases (CDH) based on Prussian blue formation in the presence of cellobiose, ferric acetate, and ferricyanide [77]. Three types of specific growth media were developed by Vasilchenko et al. (2012) for the induction of carbohydrate oxidoreductase activity. Medium 1 contains (g/l) ammonium sulfate ($(NH_4)_2SO_4$) (1.5), sodium nitrate ($NaNO_3$) (1.5), monopotassium phosphate (KH_2PO_4) (1.0), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (0.5), α-cellulose (20.0), also enriched with a mixture of xylose, arabinose, galactose, mannose and cellobiose (2.0). Medium 2 consists of milled sugar beet pulp (15.0) and malt sprouts (6.0), and medium 3 is different from medium 2 by also containing bacto-tryptone. The test strains are inoculated and incubated for 7 days, after which 5 mm × 5 mm agar slabs with fungal mycelia are cut using a sterile scalpel and transferred to a plate. Later, 20 ml of molten 2 % agar containing 0.15 mM cellobiose (or 0.21 mM of D-glucose, D-xylose, L-arabinose, D-galactose, or D-mannose) along with 0.78 mM $K_3[Fe(CN)_6]$ and 0.16 mM $NH_4Fe(SO_4)_2$ in sodium acetate buffer with pH 4.0 is sterilized and added to the plate at 42–43 °C. Once the plates are solidified, they are incubated for 2–3 hours at room temperature; plates are further observed for the development of blue color halo zones around the mycelial slabs [77].

Quantitative Assays for Cellobiose Dehydrogenase

Prussian Blue Enzymatic Reactions Vasilchenko et al. (2012) also developed an assay for quantification of CDH using cellobiose, ferric acetate, and ferricyanide, resulting in formation of Prussian blue [77]. Cellobiose dehydrogenase induces the formation of Prussian blue by reducing ferricyanide to ferrocyanide by reacting with an excess of Fe^{3+} ions. It also reduces ferric ions to Fe^{2+} by reacting with an excess of ferric cyanide [77]. The reaction mixture consists of 0.1 M sodium acetate buffer with pH of 4.5, 0.16 mM $NH_4Fe(SO_4)_2$, 0.78 mM $K_3[Fe(CN_6)]$, and 0.15 mM cellobiose. The formation of Prussian blue by CDH in the presence of 0.5–2 mM $NH_4Fe(SO_4)_2$, 2.5–10 mM $K_3[Fe(CN_6)]$ in

Table 3 Comparison of different qualitative and quantitative methods for the identification and characterization of ligninolytic peroxidases

Isolation method	Method type	Time efficiency	Cost efficiency	Method efficiency	Reference
Poly-R478 agar plate	Qualitative	+ +	$ $ $	+ +	[25, 65]
Azure B agar plate	Qualitative	+ +	$ $	+	[67]
Veratryl alcohol assay	Quantitative	+ + +	$	+ + +	[70]
Azure B assay	Quantitative	+ + +	$	+ + +	[67, 71]
Methylene blue assay	Quantitative	+ + +	$	+ + +	[68]
Methyl catechol assay	Quantitative	+ + +	$	+ + +	[69]
Phenol red assay	Quantitative	+ + +	$	+ + +	[73, 74]
Vanillyl acetone assay	Quantitative	+ + +	$ $	+ + +	[75]
DMP assay	Quantitative	+ + +	$	+ + +	[76]
Mn(II) substrate assay	Quantitative	+ + +	$	+ + +	[72]

Time efficiency = 1– 2 days (+ + +), 3–10 days (+ +), 10 or more days (+); cost per gram = $ (1–10$), $ $ (10–20$), $ $ $ (20–100 $), $ $ $ $ (100$ or more); efficiency of method in characterizing lignolytic activity = methods which can prove lignolytic activity (+ + +), methods which require further confirmation (+ +), preliminary isolation methods (+)

0.1 mM sodium acetate buffer (pH 3.0–5.5) is monitored constantly at 700 nm [77]. Similarly, Kramer et al. (1992), have shown that the reduction of (Fe^{3+}) ferric acetate spectrophotometrically in the presence of sodium acetate (0.1 mM with pH 3.0–5.0) at absorbance of 340 nm with absorption coefficient for Fe^{3+} is 1.33 mM^{-1} cm^{-1} [78]. CDH enzyme assay developed by Kramer et al. (1992) was based on reduction of ferricyanide by CDH in the presence of cellobiose (5 mM) in sodium acetate buffer (pH 2.0-5.0) by monitoring the changes in absorbance using spectrophotometer at 420 nm [78].

2,6-Dichlorphenol-Indophenol (DCPIP) From studies it is known that cellobiose dehydrogenase (CDH) oxidizes cellobiose to cellobionolactone in the presence of an electron acceptor such as DCPIP, cytochrome c, or metal ions [79]. DCPIP is a suitable electron acceptor for CDH enzyme; thus, DCPIP is widely used for the quantification of CDH enzyme activity [79]. According to Baminger et al. (2001) cytochrome c (equine), azino-di-(3-ethyl-benzthiazolin-6-sulfonic acid) cation radical are the best electron acceptors; at the same time, DCPIP, 1,4 benzoquinone, dyes such as methylene blue, phenoxazine dyes (such as Meldola's Blue and ferricyanide), and phenothiazine are also considered as efficient electron acceptors. The reaction mixture consists of 100 μl DCPIP (prepared by dissolving 3 mM in water containing 10 % v/v ethanol), 100 μl lactose (prepared by dissolving 3 mM 300 mM in 100 mM sodium acetate buffer with pH of 4.0); to the above solution appropriate amounts of same buffer, sodium fluoride, and lactose are added as specified. Reaction is initiated by adding diluted cellobiose dehydrogenase sample (20 μl) to the reaction mixture to make the total volume up to 1 ml. Reaction mixtures without sodium fluoride, laccase, and culture filtrates are considered as blanks. The reaction is immediately monitored using a spectrophotometer for the first 5 minutes at absorbance of 520 nm ($\varepsilon_{520} = 6.8 \times 103\ M - 1\ cm - 1$). In order to calculate the CDH activity, only the linear range of the slope is used; thus, one unit of enzyme activity is defined as the amount of CDH reducing 1 μmol of DCPIP per minute under standard reaction conditions [80].

DNS Assay Henrikson et al. (1997) developed an enzymatic assay for CDH which is insensitive to the laccases or other phenoloxidases which are usually present in the living systems. This method is based on the decrease of reducing end groups in lactose determined by the DNS method. Ferricyanide present in the reaction mixture acts as an efficient electron acceptor for the CDH enzyme system. The reaction is carried out in screw-cap test tubes; reaction mixture consists of 2.5 ml of cold substrate solution of 4 mM potassium ferricyanide added along with 2.5 mM lactose (in 50 mM sodium acetate buffer pH 5.0). Suitable volumes of enzyme or culture filtrate and 50 mM sodium acetate buffer (pH 5.0) are added to the reaction mixture, making up a final volume of 5 ml. The contents of the reaction mixture are mixed thoroughly and incubated at 40 °C. The 0.5 ml of test samples are taken at regular intervals from time zero (after adding CDH) and immediately mixed with 0.5 ml of DNS reagent; addition of DNS reagent stops the reaction. After collecting all the samples, tubes with DNS reagents are boiled for 5 minutes and cooled to room temperature, followed by monitoring absorbance at 575 nm. The slope of decreasing absorbance calculated from linear region is transferred to molar concentration by dividing with 752 which is the molar extinction coefficient estimated for standard curve of glucose. One unit of CDH is defined as the amount of lactose (1 nmol) oxidized per minute under standard conditions, which is determined by monitoring CDH absorbance at 280 nm [81].

Qualitative and quantitative methods described above for the characterization of cellobiose dehydrogenase enzyme are compared efficiently with respect to their cost, time and method efficiencies (Table 4).

Lignin-Degrading Auxiliary Enzymes

Apart from lignin-oxidizing (LO) enzymes, several wood-decaying fungi are also known to secrete a variety of lignin-degrading auxiliary enzymes (LDA). These LDA enzymes are required for the efficient action of LO enzymes (especially lignolytic peroxidases), by providing hydrogen peroxide. According to FOLy (fungal oxidative lignin enzymes), the

Table 4 Comparison of different qualitative and quantitative methods for the identification and characterization of cellobiose dehydrogenase

Isolation method	Method type	Time efficiency	Cost efficiency	Method efficiency	Ref
Prussian blue agar plate	Qualitative	+ +	$ $	+ +	[77]
Prussian blue enzyme assay	Quantitative	+ + +	$ $	+ + +	[77, 78]
DCPIP assay	Quantitative	+ + +	$ $	+ + +	[80]
DNS assay	Quantitative	+ + +	$ $	+ + +	[81]

Time efficiency = 1– 2 days (+ + +), 3–10 days (+ +), 10 or more days (+); cost per gram = $ (1–10$), $ $ (10–20$), $ $ $ (20–100 $), $ $ $ $ (100$ or more); efficiency of method in characterizing lignolytic activity = methods which can prove lignolytic activity (+ + +), methods which require further confirmation (+ +), preliminary isolation methods (+)

LDA are divided into seven different classes: LDA1 (aryl alcohol oxidase), LDA2 (vanillyl alcohol oxidase), LDA3 (glyoxal oxidase), LDA4 (pyranose oxidase), LDA5 (galactose oxidase), LDA6 (glucose oxidase), LDA7 (benzoquinone reductase) [8]. Different quantitative enzymatic assays used for assessing the enzyme activity of LDAs are discussed below (Fig. 3).

Aryl Alcohol Oxidase

Veratryl Alcohol Assay Several studies have reported the usage of veratryl alcohol as substrate for the quantification of aryl alcohol oxidase (AAO) [82–89]. The reaction mixture consists of 1 mM veratryl alcohol in 0.05 M citrate phosphate buffer (pH 3.0), purified enzyme or culture filtrate making the total volume up to 2 ml, which is used for the determination of oxidase activity. The oxidation reaction of the veratryl alcohol at room temperature is monitored constantly at an absorbance of 310 nm ($\varepsilon_{310} = 9{,}300\ M^{-1}\ cm^{-1}$); the resulting increase in absorbance is due to the formation of veratraldehyde. The enzyme activity is measured by the amount of the enzyme that converts 1 μmol of veratryl alcohol to veratraldehyde (product) obtained per minute [82–88].

Vanillyl Alcohol Oxidase Studies have shown that vanillyl alcohol oxidases (VAO) catalyze the oxidation of various aromatic substrates, mainly 4-hydroxybenzyl alcohols vanillyl alcohol, eugenol, chavicol, 4-alkyl-phenols [90, 91]. Fraajie et al. (1995) showed the substrate specificity for VAO, which conveys that apart from oxidation of 4-hydroxybenzyl alcohols, VAO can also catalyze the oxidative deamination and demethylation of 4-hydroxybenzylamines and 4-methoxymethyl phenols respectively (Table 5). It is believed that vanillyl alcohol is the only enzyme inducer and growth substrate for VAO, thus named as vanillyl alcohol oxidase. Furukawa et al. (1999) proposed that VAO showed higher affinity and enzyme activity for eugenol when compared to vanillyl alcohol. Vanillyl alcohol was converted to vanillin with much lower affinity, and only 50 % of enzyme activity was observed when compared to eugenol [90].

(i) Vanillyl alcohol oxidase is routinely assayed using vanillyl alcohol by producing vanillin, which can be monitored using a spectrophotometer at 340 nm [92]. The reaction mixture contains a total volume of 2.4 ml containing culture filtrates (dialyzed and cell free), 115 mM $(NH_4)_2SO_4$, 42 mM glycine/sodium hydroxide (pH 10.0). The reaction is initiated by adding 1.25 mM vanillyl alcohol to the reaction mixture; one unit of enzyme oxidizes 1 μmol vanillyl alcohol to vanillin at pH 10.0 and 30 °C [92, 93].

(ii) Furukawa et al. (1999) used eugenol for the first time to perform the enzymatic assay of VAO. The reaction mixture contains 10 mM eugenol and a suitable amount of enzyme filtrate in 1 ml of buffer incubated at 30 °C in a shaker at 160 rpm. The reaction is stopped by adding 1 ml of methanol, and then the formation of coniferyl

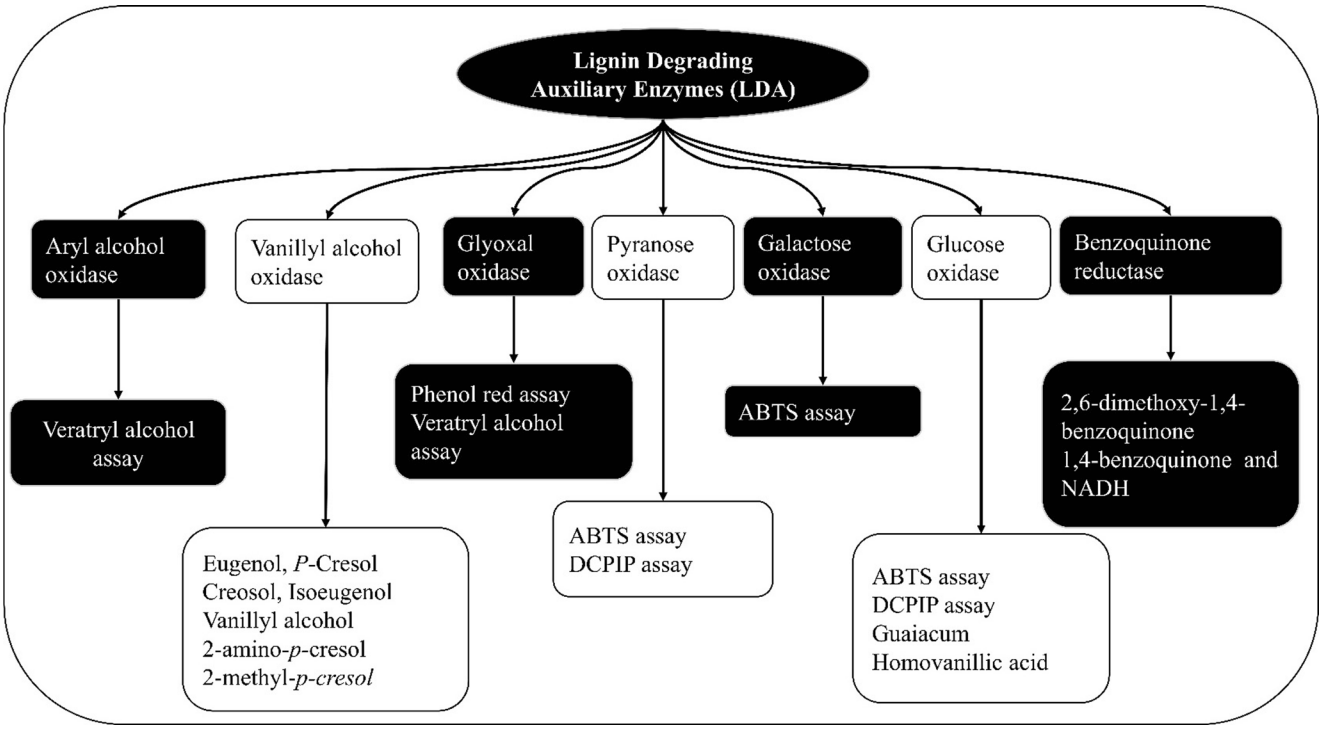

Fig. 3 Different enzymatic assays used for the quantitative estimation of lignin-degrading enzymes (LDA)

Table 5 Different substrates of vanillyl alcohol oxidase (VAO) and their quantitative enzymatic assay conditions

Substrate	pH	Absorbance (nm)	Product
Eugenol	7.5	296 nm	Coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol)
(4-allyl-2-methoxyphenol)	10	314 nm	
p-cresol, (4-methoxymethyl phenol)	7.5	330 nm	4-hydrobenzaldehyde (for *p*-cresol)
Creosol (2-methoxy-4-methylphenol)	7.5	340 nm	Vanillin
Isoeugenol (2-methoxy-4-propenylphenol),	7.5	290 nm	4-hydroxycinnamyl alcohol (for isoeugenol)
Vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol)	7.5	340 nm	Vanillin
2-amino-*p*-cresol	7.5	340 nm	2-amino-4-hydroxybenzaldehyde
2-methyl-*p*-cresol	7.5	340 nm	2-methyl-4-hydroxybenzaldehyde

alcohol is confirmed by HPLC. The enzyme activity of VAO is defined as the amount of enzyme catalyzing oxidation of eugenol for the production of 1 μmol coniferyl alcohol per minute [90].

(iii) Van den Heuvel et al. (2004) have used a wide range of aromatic substrates for the quantitative estimation of VAO enzyme activity [94]. The standard enzymatic assays were performed using a set of aromatic substrates which are shown in Table 5. Van den Heuvel et.al (2004) have determined the enzymatic yield conversion using HPLC analysis, and the usual enzyme activity is constantly monitored using spectrophotometer at different absorbances (nm) (mentioned in Table 5). The reaction mixture consists of 8 μM enzyme or culture filtrate and 200 μM air saturated substrate, and the redox state of the FAD (flavin adenine dinucleotide) cofactor is continuously monitored using a spectrophotometer [94].

Glyoxal Oxidase

H_2O_2-Producing Activity Using Phenol red The production of hydrogen peroxide is determined by a modified peroxidase coupled assay using phenol red as peroxidase substrate [95]. The reaction mixture contains 50 mM sodium 2–2, dimethylsuccinate (pH 6.0), 10 mM methylglyoxal (oxidase substrate), 0.01 % phenol red, and 10 μg of horseradish peroxidase, and finally the reaction is initiated by adding 300 μl of culture filtrate making total mixture up to 1 ml. Reaction is stopped by adding 50 μl of 2 N sodium hydroxide; the reaction is constantly monitored at 610 nm using an appropriate control. The optimum pH for the oxidase activity is maintained using buffer containing 25 mM 2–2, dimethyl succinate, and 25 mM 3-(N-morpholino) propane sulfonic acid, and then the pH is adjusted using sodium hydroxide. The enzyme activity is determined by measuring the H_2O_2 produced by glyoxal oxidase in the oxidization of methylglyoxal; a calibration curve is drawn with increasing concentrations of H_2O_2 from 0 to 60 μM. Glyoxal oxidase activity is indicated by measuring the H_2O_2 produced in nanomoles per minute per millilitre [73, 95].

Veratryl Alcohol A previous assay method used to detect activity of cultures using commercially available peroxidase [95]. Veratryl assay was developed to minimize the anomalies raised during enzyme activity, such as lags and activations becoming obvious when purified glyoxal oxidase was used; thus, conditions for improving approximates of cultures were used for characterizing the oxidase and peroxidase interaction systems. The reaction mixture contains 20 mM sodium 2,2-dimethylsuccinate (pH 4.5), 5 mM methylglyoxal, and 2 mM veratryl alcohol, and finally glyoxal oxidase and glyoxal oxidase free lignin peroxidase is added (non-rate limiting). The reaction is constantly monitored by a spectrophotometer at 310 nm for observing the veratraldehyde formation. In order to eliminate the lags in the enzyme activity caused by purified glyoxal oxidase, 5 μM hydrogen peroxide is added, resulting in a rapid uncoupled reaction of lignin peroxidase which consumes the exogenous hydrogen peroxide. Thus, the slower and extensive reaction of glyoxal oxidase is measured using spectrophotometer [96].

Pyranose Oxidase

ABTS Assay Pyranose oxidase (PO) is routinely assayed based on the stoichiometric formation of hydrogen peroxide upon substrate oxidation reactions. The hydrogen peroxide obtained is detected by peroxidase catalyzed chromogenic reaction using ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) as a substrate at an absorbance of 420 nm. The reaction mixture contains 100 μmol potassium phosphate (pH 6.5), 1 μmol of ABTS, 2U of peroxidase, and 5–20 mU pyranose oxidase, making the total volume up to 1 ml at 30 °C. Reaction is initiated by adding 100 μmol of sugar substrate, preferably glucose. The reaction is continuously monitored using a spectrophotometer at 420 nm; two molecules of ABTS are oxidized per single molecule of reduced

hydrogen peroxide. The enzyme activity is defined as one unit of pyranose oxidase required for the oxidization of 2 μmol ABTS under the standard assay conditions [89].

2,6-Dichlorophenolindophenol (DCPIP) Assay DCPIP is a well-known two-electron acceptor for various flavin-dependent oxidoreductases; pyranose oxidase is usually quantified by performing a time-dependent reduction of DCPIP substrate. The reaction mixture consists of 150 μM DCPIP in 50 mM phosphate buffer (pH 6.5), 20 mM D-glucose substrate at 30 °C. The reaction is initiated by adding 10 μl of diluted pyranose oxidase enzyme or an appropriate amount of culture filtrate; decolorization of the substrate is continuously monitored using a spectrophotometer at an absorbance of 520 nm. The enzyme activity is defined as one unit of pyranose oxidase required for the decolorization (oxidization) of DCPIP per minute [97].

Galactose Oxidase Galactose oxidase activity is conventionally monitored by peroxidase-coupled ABTS reaction. Horseradish peroxidase (HRP) catalyzes the oxidization of ABTS substrate by taking up hydrogen peroxide released by galactose oxidase, resulting in a soluble green-colored end product which can be measured using a spectrophotometer at 410 nm. The reaction mixture consists of 8 mg D-galactose, 20 mg of ABTS substrate, 300 units or 3.3 mg of horseradish peroxidase dissolved in 20 ml of 100 mM sodium phosphate buffer at pH 7.0. According to Baron et.al (1994), this method can be used both qualitatively and quantitatively for the estimation of galactose oxidase [98]. Qualitative assays for galactose oxidase contain 90 μl of reagent and 10 μl of culture filtrate or enzyme solution added to each well and kept aside. The plates are constantly observed for the development of green color; this test is a convenient method for preliminary analysis of culture medium. Reaction mixture of quantitative assay contains 50 μl culture filtrate or enzyme mixed with 1.0 ml of reagent, and the reaction is continuously monitored using spectrophotometer at 410 nm [98].

Glucose Oxidase Glucose oxidase (GOX) is a highly studied enzyme for its applications in modern medicine. It is known that fungi and insects are the major sources of glucose oxidase enzyme. GOX also plays a critical role during the biomass degradation process of fungi by generating hydrogen peroxide. Different dyes can be used for the quantitative estimation of GOX activity, such as *o*-Dianisidine [99, 100], ABTS, 2,6-Dichlorophenolindophenol (DCPIP) [100], *o*-toluidine[101], guaiacum [102], 4-aminophenazone or adrenaline [103], 3-methyl-2-benzothiazolinone hydrazone coupled with N,N–dimethylaniline [104], N,N-diethylaniline oxidatively coupled with 4-aminophenazone or phenol [105], and leuco Patent Blue Violet [106]. Chemical properties of ABTS such as solubility, safety, and stability makes it highly advantageous when compared to all the other chemical substrates. Conventionally, GOX is quantified using the peroxidase–glucose oxidase coupled reaction, where glucose and oxygen form as a substrate for GOX. The glucose oxidase ABTS assay reaction mixture consists of 1 ml of 100 mM sodium phosphate buffer (pH 6.0), 0.5 ml of 1 M glucose, 0.1 ml of 1 mg/ml of ABTS in distilled water, and finally 0.1 ml of 2 mg/ml horseradish peroxidase (HRP) in sodium phosphate buffer (pH 6.0). The oxidized product of ABTS forms a green-colored product which can be continuously monitored using spectrophotometer at 414 nm. One unit of enzyme activity is defined as the amount of GOX required for the oxidization of 1 μmol of ABTS per minute at standard conditions [107, 108].

Fluorometric Method Using Homovanillic Acid Guilbault et al. (1968) have proposed a new fluorometric method using homovanillic acid for determining the activity of oxidative enzymes such as peroxidases and glucose oxidases [109]. This method involves the conversion of non-fluorescent homovanillic acid to fluorescent 2,2'-dihydroxy −3,3'-dimethoxybiphenyl-5,5'-diacetic acid; the rate of formation of fluorescent compound is related to the activity of the enzyme [109]. The reaction mixture contains 2.7 ml of 0.1 M tris buffer (pH8.0) mixed with 0.1 ml of stock solution, 0.1 M glucose solution, 0.1 ml of 2.5 mg/ml homovanillic acid, and 0.1 ml of 0.75 mg /ml peroxidase solution (HRP). The reaction is initiated by adding 0.1 ml of glucose oxidase solution to the reaction mixture; the whole reaction is continuously monitored for change in fluorescence using a spectrophotofluorometer [109].

Benzoquinone Reductase The enzymatic activity of benzoquinone reductase can be performed by two different methods, using 2,6-dimethoxy-1,4-benzoquinone (2,6-DMBQ) or 1,4-benzoquinone and NADH [110–112]. Enzyme activity of quinone reductases is routinely assayed using 2,6-DMBQ; this method estimates the oxidation of NADH by quinone reductase. The reaction mixture using 2,6-DMBQ contains 1 ml of 50 mM sodium citrate buffer with pH 6.0, 100 μM 2,6-DMBQ, and benzoquinone reductase enzyme. Reaction is initiated by adding 200 μM NADH to the reaction mixture at room temperature. The whole reaction is monitored using a spectrophotometer at 340 nm [110]. Benzoquinone reductase enzyme activity can also be assessed quantitatively using 1,4-benzoquinone, where the reaction mixture consists of 25 mM Tris–HCl buffer with pH 8.0, 250 μM 1,4-benzoquinone, 200 μM NADH, and benzoquinone reductase. Reaction is initiated by the addition of NADH; the total reaction is monitored using spectrophotometer at 340 nm [111]. Constam et al. (1991) used structurally different quinones for assaying quinone reductase enzyme [112]; this assay consists of 25 mM Tris/HCl buffer (pH 8.0), 250 μM of different quinone structures (QI, QII, QIII and QIV) [112], and 200 μM of NADH,

and an appropriate amount of enzyme is added. The whole reaction is continuously observed at 340 nm using spectrophotometer; this method has shown the non-enzymatic oxidization of NADH by quinones. The enzyme activity is defined as one unit of quinone reductase required for the oxidization of 1 μM of NADH per minute [112].

In order to gain a complete understanding about the lignin-degrading abilities of the microorganisms, it is important to comprehend the underlying molecular pathways required for the expression of lignin-degrading enzymes. At the same time, it is also equally important to study the functional and structural properties of these ligninolytic enzymes, which can be achieved by employing various well-established protein and gene characterization techniques. A number of research articles on gene and protein characterization techniques are available, and a few are listed below to indicate the standard protocols [89, 90, 96, 110, 113–119].

Concluding Remarks

The intricate structure of lignin can potentially be a valuable source for the production of commercially valuable platform chemicals. Efficient utilization of lignin and other cell-wall polysaccharides such as cellulose and hemicellulose are significantly dependent on their efficient separation. Traditional methods for the isolation and characterization of lignin-degrading microorganisms were already well established. However, selecting an efficient method for qualitative and quantitative characterization of lignin-degrading microbes and their respective ligninolytic enzymes is difficult. This article intensively reviews all the reported methods up to the present, and compares the advantages and disadvantages of each method. It also provides useful guidelines for selecting the appropriate isolation and characterization methods based on their cost, time, and method efficiencies. In the past decade, there has been a tremendous advancement in the field of genomics, especially next-generation sequencing. The whole genome sequences of prominent wood-degrading fungi such as *P. chrysosporium* and *Postia placenta* have revealed high-quality information about their lignocellulose-degrading abilities on a large scale [120, 121]. These studies have resulted in a rapid increase in whole genome and transcriptome studies of various wood-decaying microorganims using DNA, RNA, and ChIP sequencing protocols [122, 123]. Thus, well-designed experiments with a combination of traditional low throughput and recent state of the art high-throughput methods will be powerful in identifying and characterizing lignin-degrading microorganims and their molecular pathways.

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