Screening and Enzymatic assays of potent Cellulolytic andLignolytic Rot Fungus

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Abstract:
The fungal enzymes contribute to around 40% of global market of enzymes (Sudarson *et al.*, 2014). Screening for enzyme production by different species of fungi which were isolated from different regions of Western Ghats of Karnataka based on their physiological characteristics and pattern of wood decay is important to categorize them according to the specific functions and usage. The enzymes which are produced from plant biomass by the fungal species possess wide range of application in the field of medicine, textile, bio-fuel production and research. Wood decaying class of fungi including white rot, brown rot and soft rot species prefer to grow in moist (35-50%), woody ecosystem and utilize different metal ions including Mn, Fe, Cu for their energy metabolism and enzyme production (Ringman *et al.*,2019). Scientists have reported that the enzymes which degrade lignin are efficiently capable to degrade polychlorinated biphenyls, polycyclic aromatics hydrocarbons (PAHs), pesticides,and dyes (Lee *et al.*, 2020). Several enzymatic assays are actively performed as confirmation tests to both quantitative and qualitatively detect the lignin degrading capacity of selected fungi. Out of 100 collected samples from different regions of Western Ghats in Karnataka 50 samples were categorized and studied further for their capability of producing lignin and cellulose degrading enzymes and only 25 such samples were considered further as potent enzyme producing fungi which can effectively degrade lignocellulosic materials.
Key Words: Plant biomass, Wood rot fungi, PAHs, Ligninase enzyme, polychlorinated biphenyls, lignocellulosic.

Introduction

Elucidating the enzymatic mechanisms of wood rot fungi is not only about their metabolism and carbon cycle, but also because these fungal species are industrially and economically important due to their capability of producing biomass for various industrial process and energy sources (Hussin, 2018). The enzymes which are produced from plant biomass by the fungal species possess wide range of application in the field of medicine, textile, bio-fuel production and research. The fungal enzymes contribute to around 40% of global market of enzymes (Sudarson *et al.*, 2014). Screening for enzyme production by different species of fungi which were isolated from different regions of Western Ghats of

Karnataka based on their physiological characteristics and pattern of wood decay is important to categorize them according to the specific functions and usage. It is reported that degradation of lignin, cellulosic materials are carried out largely by white rot, brown rot and soft rot fungi from phylum Ascomycota and Basidiomycota (Leonhardt *et al.,* 2019). Studies have suggested that some white rot fungi are very specific to lignin degradation where as some other species of white rot fungi can degrade both the lignin and some polysaccharides (Madadi & Abbas, 2017). Brown rot fungi cannot degrade lignin as they do not possess lignin degrading enzymes, however they can efficiently degrade cellulosic materials (Su *et al.,* 2018). Studies have reported that to degrade lignocellulosic materials the wood rot fungi which produce lignocellulolytic enzymes use a part of their metabolic energy. The ability of fungal species to secrete potential hydrolytic and oxidative enzymes regulates their capability of utilizing lignocellulosic materials and the secretion of such enzyme varies species to species (Andlar *et al.,* 2018). Due to such diversity at molecular level, each fungi sample is required to be analyzed qualitative and quantitatively for the production of specific enzymes under specific growth conditions.

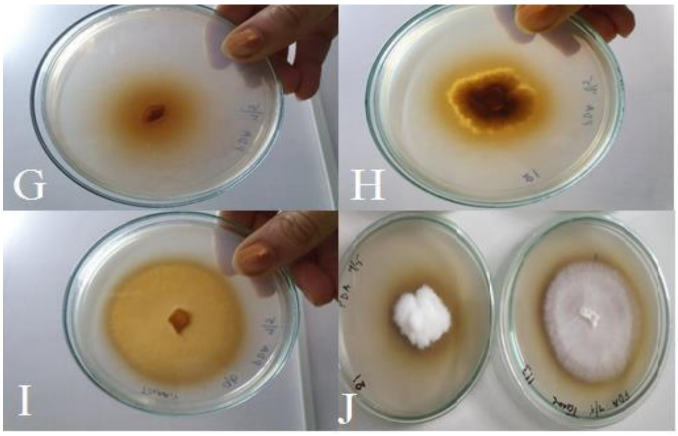
Wood decaying class of fungi including white rot, brown rot and soft rot species prefer to grow in moist (35-50%), woody ecosystem and utilize different metal ions including Mn, Fe, Cu for their energy metabolism and enzyme production (Ringman *et al.,* 2019). But growing them in laboratory on specific growth media for screening based on the enzyme production is little tricky. However, the growth condition for screening the fungi based on enzyme production is experimentally standardized (Wang *et al.,* 2019). The qualitative assay for detection of presence of cellulose degrading enzyme in the fungal isolate is studied by growing them on basal media (BSM) with presence of 2% carboxy methyl cellulose and congo red dye. Based on the zone of clearance the qualitative screening of enzyme producing fungi is done (Pingili *et al.,* 2017). Similarly, detection of lignin degrading enzyme such as laccase (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP) and lignin depletion is studies by different methods including alkali lignin removal rate assay, radioactive carbon-14 (^{14}C) isotope assay. There are different methods such as dye de-coloration assays, agar plate assays, staining assays, genomics andproteomics assays, gel permeation, ion exchange chromatography assays, methylene blue assay, phenol red assay etc. for qualitatively and quantitatively characterize the cellulose and lignin degrading enzyme producing fungi (Kameshwar *et al.,* 2017). Scientists have reported that the enzymes which degrade lignin are efficiently capable to degrade polychlorinated biphenyls, polycyclic aromatics hydrocarbons (PAHs), pesticides,and dyes (Lee *et al.*, 2020). Several enzymatic assays are actively performed as confirmation tests to both quantitative and qualitatively detect the lignin degrading capacity of selected fungi. In this context, the presence of laccase, manganese peroxidase and lignin peroxidase are also analyzed by qualitative and quantitative methods. Lignin peroxidase is a glycoprotein in which the heme group is more electro deficient than other peroxidases and results in higher redox potential. Thus, eventually contributes in oxidizing of a range of aromatic compounds such as veratryl alcohol, methoxybenzenes which are not susceptible to other peroxidases. Activity of lignin peroxidase can be monitored by Azure B assay (Casciello *et al.,* 2017). As the compound shows higher specificity with respect to lignin peroxide activity. Manganese peroxide is a heme peroxidase which are capable of one electron oxidation and the enzyme gets stabilized with the help of Mn^{3+} chelator complex. This chelator complex cannot degrade phenolic compounds. As a result, at pH 4.5 the manganese peroxidase can depolymerize and repolymerize lignin substances.The activity of manganese peroxidase can be determined by phenol red assay. Laccasesare monomeric polyphenol oxidases. They can only oxidize phenolic substances including polyphenols, methoxy-substituted phenols, and aromatic diamines due to their relatively low redox potential. As laccases possess a broad level of substrate specificity, determination of their activity is difficult. ABTS [2, 2'-azinobis-(3-ethylbenzthiazoline-6- sulfonate)] assay shows promising method for determination of laccase activity (Dias *et al.*,2017).

Cellulases are the example of multi-unit enzymes which are made up of mainly three major groups such as 1,4-β-d-endoglucanase (EC 3.2.1.4), 1, 4-β-d- cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). These three groups of enzymes functions synergistically to hydrolyze cellulose into simple sugar (Tiwari *et al.*, 2013). Out of 100 collected samples from different regions of Western Ghats in Karnataka 50 samples were categorized and studied further for their capability of producing ligninand cellulose degrading enzymes and only 25 such samples were considered further as potent enzyme producing fungi which can effectively degrade lignocellulosic materials. Fungal cellulases are also industrially important as they are extracellular and can be produced in bulk. But in all the cellulose degrading fungi do not possess active cellulose;as a result, they cannot degrade cellulose efficiently. Hence, for detection of active cellulase producing fungi which can effectively degrade cellulose qualitative and quantitative analysis of their cellulase production is necessary (Yarkwan & Anyanwu, 2016).

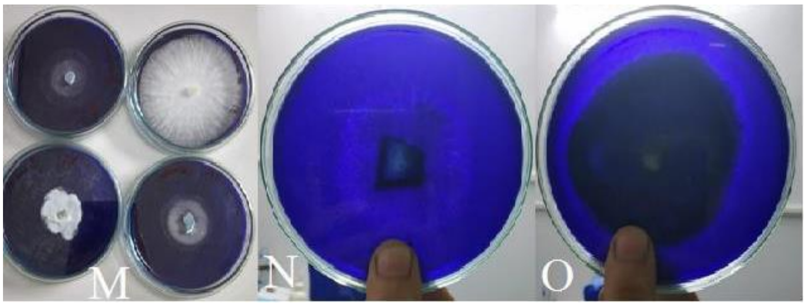
Materials and Methods

Qualitative Screening for lignin degrading fungi

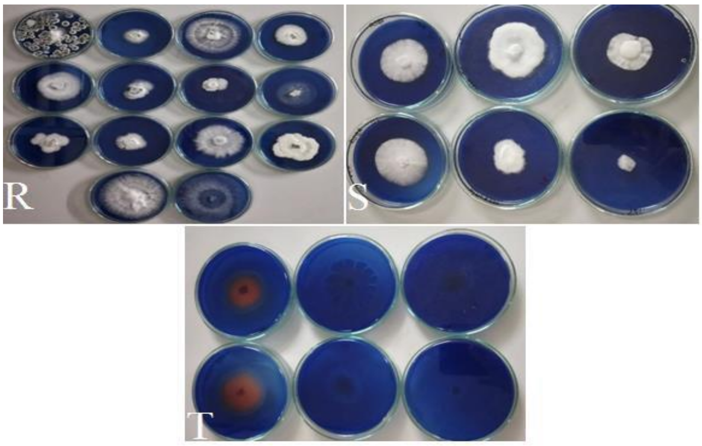
The fungal isolates were screened for lignin degrading enzymes by growing them on the solid agar plates of selected dyes (Sharma *et al.*, 2017).



Rot fungus growth on Tannic Acid Agar and production of dark brownzone around the colonies



Dorsal view of Rot fungus growth on Azure-B agar media and zone of clearance around the colony, N and O- ventral view with zone of clearance.



Dorsal view of Rot fungus growth and decolourization on Malt extract agar with Remazol brilliant blue.T- ventral view of mycelialgrowth on Malt extract agar with Remazol brilliant blue.

Dorsal view of Rot fungus growth on CMC media. Z, A1, B1 and C1-Dorsal view of Rot fungus growth and Zone of clearance on CMC agar media.

Tannic Acid Agar test

For Tannic acid agar test (Table 2.1), 7-day old culture was used, 8 mm diameter plugs were inoculated onto sterile tannic acid agar plates containing 0.2% tannic acid and incubated at 30 °C. Growth was followed for a period of 7-10 days. Positive reaction is indicated by the formation of a light to dark brown zone around the colony. Positive results indicate the production of laccase enzyme which oxidizes tannic acid. The zones were measured and the results tabulated.

Azure –B test

For degradation and decolourization of Azure-B dye, Potato Dextrose Agar (PDA) was prepared and 0.01% of Azure-B dye (Himedia) was added and the Media was sterilized autoclaving. The sterile media was poured into sterile petri plates and allowed to solidify. The sterile media plates were inoculated with 8mm diameter agar plugs of 7 days old fungal culture. The plates were incubated at 30 °C for 7 to 10 days. Positive reaction is indicated by the formation of a clear zone around the colony. Positive results indicate the production of lignin peroxidase enzyme which decolorizes Azure B. The zones were measured and the results were tabulated.

Remazol brilliant blue-R decolourization

For degradation and decolourization of Remazol brilliant blue-R (RBBR) dye, Malt extract agar (MEA) was prepared and sterilized, 0.005 g/L Remazol brilliant blue-R (Hi media) was added and plates prepared. The sterile media plates were inoculated with 8mm diameter mycelial plugs of 7 days old culture mycelia. The plates were incubated at 30 °C in dark for 7 to 10 days until a zone of decolourization or halo around the growth of the colony in the medium was observed. The results were observed and tabulated.

CMC agar test

Screening for cellulose degradation by standard Carboxy Methyl Cellulose (CMC) agar method was carried out with all the isolates according to (Pointing *et al.* 1999).

Actively growing 7 days old culture was used to inoculate the medium, 8mm diameter plug was inoculated into sterile media plates and incubated at 30 °C for 7 days. After growth, the plates were flooded with 2% (w/v) aqueous congo red and left for 15 minutes. The stain was removed and the agar surface was washed with distilled water, the plates were then flooded with 1M NaCl to de-stain for 15 minutes. CMC degradation around the colonies appeared as a yellow zone against the red colour of undegraded CMC. The zones were measured and recorded.

The organism which showed maximum activity in the qualitative screening for dye degradation was further used in the quantitative estimation of lignin degrading enzymes.

Quantitative estimation of lignin-degrading enzymes

For quantitative screening of lignin degrading enzymes, the methodology followed was as per (Paterson & Bridge, 1994).

Actively growing 7 days old culture was used to inoculate the medium (Table 2.4)

, 8mm diameter plug was inoculated into sterile broth media and incubated at room temperature at 140 rpm for 7 to 10 days till visible growth of the mycelia was evident. After incubation the medium was filtered using Whatman filter paper and the supernatant was centrifuged at 10, 000 rpm for 10 minutes in an ultra-cooling centrifuge (REMI India). The supernatant was taken and used for further lignolytic enzyme assay.

Lignin Peroxidase

Lignin peroxidase assay was performed by taking 2.2 ml supernatant in a test tube, 0.1ml of 1.2 mM methylene blue and 0.6 ml of 0.5 M sodium tartrate buffer (pH 4.0) were added. The reaction was started by the addition of 0.1ml of 2.7 mM H_2O_2. The absorbance was measured at 664 nm (Magalhaes *et al.*, 1996)

Manganese Peroxidase

The assay solution contained 1ml of 1mM guaiacol, 1ml of 1mM $MnSO_4$ in 10mM citrate phosphate buffer (pH 5.5). The reaction was initiated by the addition of 1 ml of 50µM H_2O_2. After incubation at room temperature for 10 mins, the change in the absorbance due to oxidation of guaiacol was monitored at 465nm (Bonnen *et al.*, 1994).

Laccase activity

The assay solution contained 1 ml of 2mM guaiacol, 3 ml of 10 mM sodium acetate buffer and 1 ml of supernatant. After incubating for 15 mins at 30 ºC the absorbance was noted at 450 nm **(**Kalra *et al.*, 2013**)**.

Versatile peroxidases

The reaction mixture contained 0.5 ml of supernatant, 0.5 ml of 0.1mM $MnSO_4$, 0.5 ml of 0.1mM H_2O_2, 0.5 ml of 0.01% (w/v) phenol red, 0.5 ml of 25mM lactate, 0.5 ml of 0.1%(w/v) bovine serum albumin and 20mM sodium succinate buffer (pH 4.5). After incubation the reaction was terminated by addition of 50µl of 10% NaOH. Absorbance was measured at 610nm **(**Salame *et al.*, 2012**)**.

Cellulase Assay

The reaction mixture contained 0.2 ml of supernatant, 1.8 ml of 1% CMC, incubated at 40 ºC for 30 mins. After incubation 3 ml of DNS reagent was added and incubated again at 90 ºC for 15 mins. Allowed to cool and 1ml of Sodium potassium tartrate was added and absorbance was noted at 575 nm (Miller, 1959).

Protein estimation

For 1ml of supernatant, 4.5 ml of reagent I was added, incubated for 10 mins at room temperature. 0.5 ml of reagent II was added and incubated for 30 mins in dark. The absorbance was noted at 660 nm (Lowry *et al.*, 1951).

The enzyme assay was conducted for the isolate which showed maximum qualitative activity and the data was recorded.

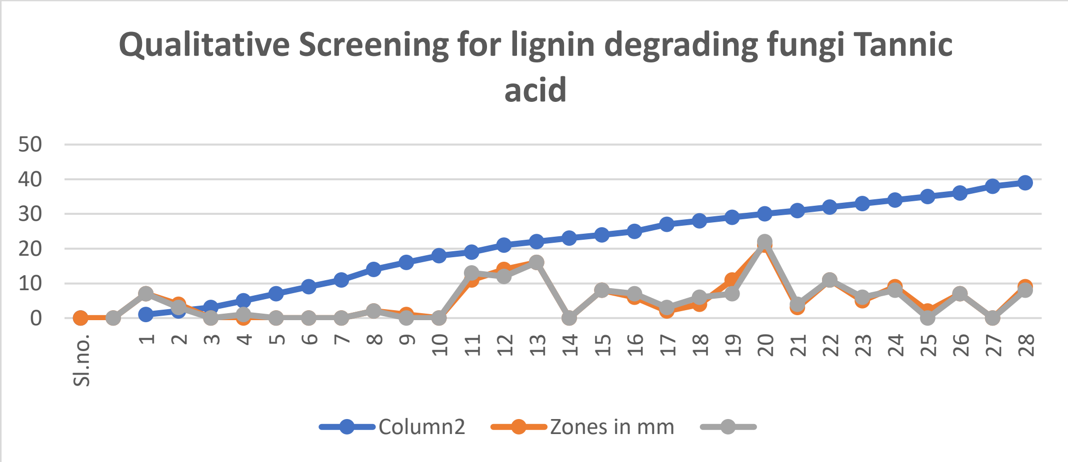
Result and Discussion

2.3.1. Qualitative Screening for lignin degrading fungi

The fungal isolates were screened for lignin degrading enzymes by growing them on the solid agar plates of selected dyes.

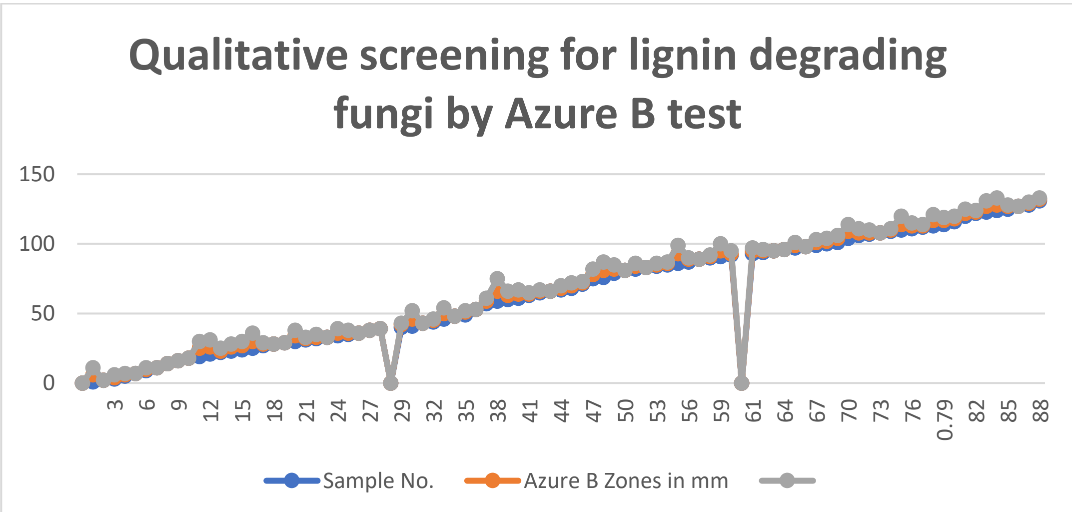
Tannic Acid Agar test

Positive reaction is indicated by the formation of a light to dark brown zone around the colony. Positive results indicate the production of laccase enzyme which oxidizes tannic acid. The zones were measured and the results tabulated.



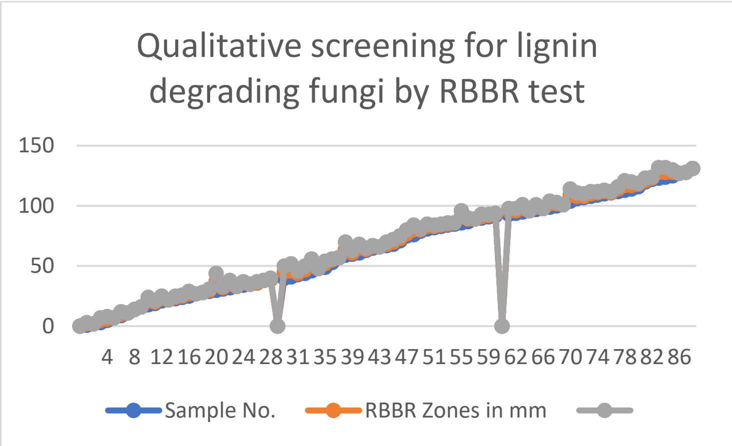
Azure –B test

Growth was followed for a period of 2 weeks. Positive reaction is indicated by the formation of a clear zone around the colony. Positive results indicate the production of lignin peroxidase enzyme which decolorizes Azure B. The zones were measured and the results tabulated.



Remazol brilliant blue-R decolourization

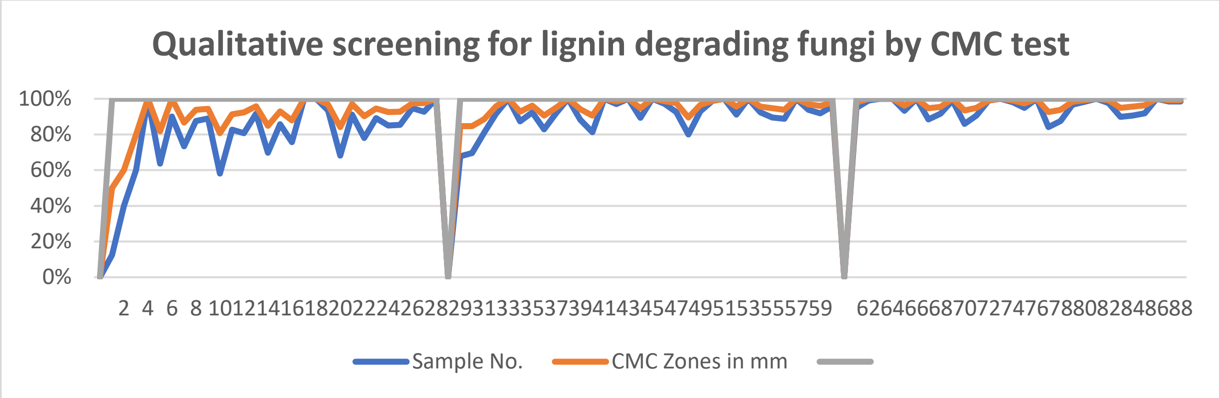
For degradation and decolourization of RBBR dye, Malt extract agar (MEA) was prepared and autoclaved, 0.005g/L Remazol brilliant bule-R (Hi media) was added and plates prepared. The plates were incubated at 30 °C in dark until a zone of decolourization or halo around the growth of the colony in the medium was observed.



CMC agar Test

Screening for cellulose degradation by standard CMC agar method was carried out with all the isolates according to Pointing *et al.* 1999. Positive result is indicated by the appearance of yellow-opaque area against the red colour of the un-degraded CMC.

Of the 88 Rot fungal strains subjected to qualitative lignin degradation It was observed that 88 fungal strains numbering 1, 19, 21, 24, 25, 30, 32, 34, 40, 41, 60, 61, 75, 76, 86, 87, 91, 93, 97, 99, 104, 106, 110, 112, 113, 123, 124, 125 exhibited lignin degrading enzymes for all the assays i.e. They were found to be positive for the production of Laccase enzyme in the oxidation of Tannic acid, in the degradation and decolourization of RBBR dye and also the production of Cellulase enzyme for the degradation of CMC



Quantitative estimation of lignin-degrading enzymes

For quantitative screening of Lignin degrading enzymes, the methodology followed was as per (Paterson & Bridge, 1994). 28 isolates which showed the maximum zone of activity against the dyes were used for the quantitative screening of enzymes. Actively growing 7 days old culture was used to inoculate the medium, 8mm diameter plug was inoculated into sterile media plates and incubated at room temperature at 140rpmfor 7 to 10 days till visible growth of the mycelia was evident. After incubation the medium was filtered using Whatman filter paper and the supernatant was centrifuged at 10, 000 rpm for 10minutes in an ultra-cooling centrifuge. The supernatant was taken and used for further ligninolytic enzyme assay.

Lignin Peroxidase

Lignin peroxidase activity was assayed by Azure-B test for 28 samples. The reaction was started by the addition of 0.5ml of 2mM H_2O_2. The absorbance was measuredat 651nm

Manganese Peroxidase

Manganese Peroxidase activity was assayed for 28 samples. The absorbance due to oxidation of guaiacol was monitored at 465nm

Laccase activity

Activity of laccase was assayed by reading absorbance at 450 nm (Figure 2.8) and activity was expressed at colorimetric units ml^{-1} ($CU\ ml^{-1}$).

Versatile peroxidase

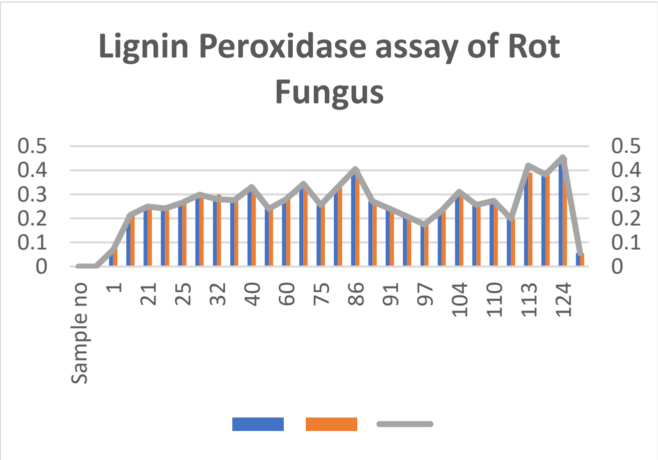
Versatile peroxidase activity was assayed for 28 samples and absorbance was measured at 610 nm.

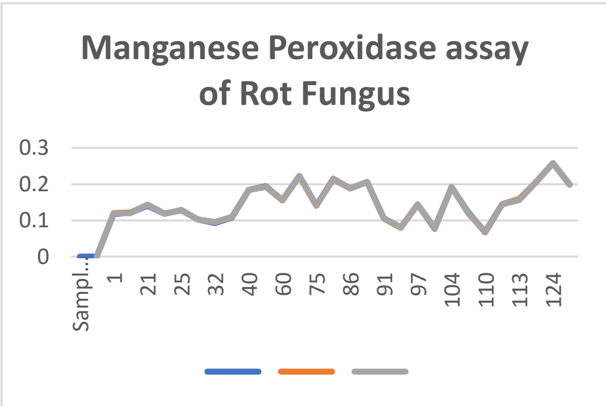
Protein estimation

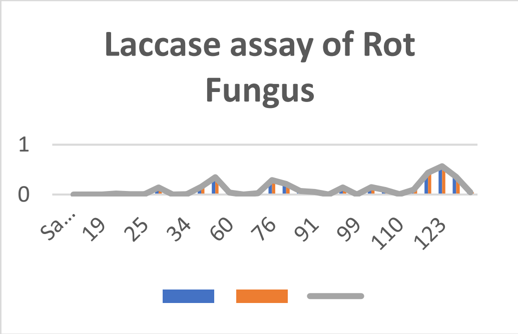
Protein estimation activity was assessed for 28 samples by reading the absorbance at 660nm (Figure 2.10). The results were tabulated as in (Table 2.14).

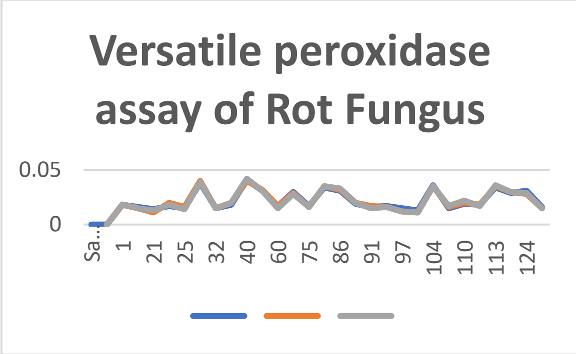
Cellulase activity

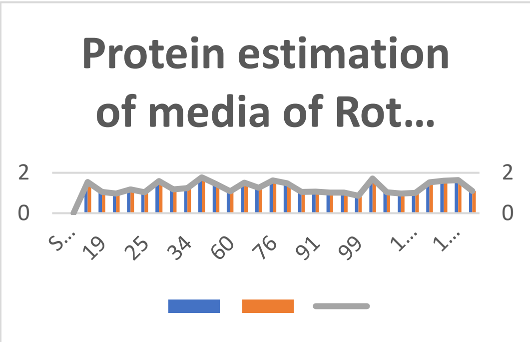
Cellulase activity was measured for 28 samples by reading absorbance at 575nm

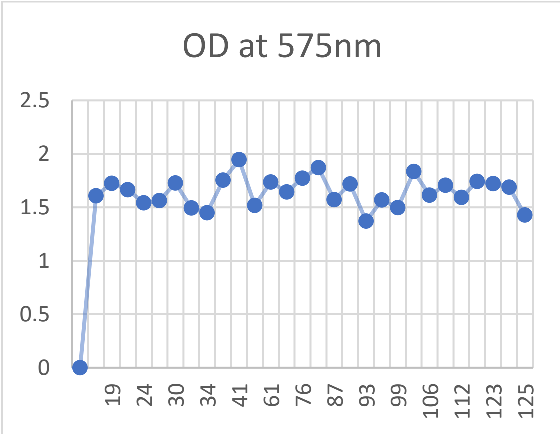












Cellulase assay of Rot Fungus

Of the 28 rot fungal strains subjected to individual quantitative estimation oflignin-degrading enzyme assay 10 strains 30, 40, 41, 61, 76, 86, 104, 113, 123 and 124 exhibited maximum activity for all the assay i.e., Lignin Peroxidase, Manganese Peroxidase, Laccase enzyme assay, Versatile peroxidase, Protein estimation and Cellulase activity. These fungal strains were further used for Fermentation and subjected to RAPD analysis to determine the genetic diversity amongst the species and identification of the same.

Rot fungi has the ability to degrade a broad spectrum of structurally diverse aromatic compounds like several environmental pollutants (Gupta *et al.*, 2018). The lignin degradation by rot fungi is a non-specific mode (Janusz *et al.*, 2017). The present work reveals that the selected rot fungi cultured in optimized conditions are useful for the excessive production of lignin modified enzymes. Veratryl alcohol found to have the ability to protect the enzymes from hydrogen peroxide dependent inactivation and thus to increase in enzyme production (Selvam *et al.*, 2012) increased enzyme production by using veratryl alcohol as a mediator was observed in this study.

Discussion

The fungal isolates were screened for lignin degrading enzymes by growing them on the solid agar plates of selected dyes quantitatively by measuring zone surrounding the colony.

Total 88 fungal species samples were tested for Laccase production (activity)/ability to oxidize tannic acid. Sample No. 113 showed highest Laccase activity by producing a zone of 26.67mm followed by sample no.104, 61, 86, 76, 30, 40, 123, 124, 108, 41, 22, 13, 21, 19 and 125. Strain number 3, 7, 18, 38, 43, 53,59, 63, 94, 96 and 128 absolutely showed no/or zero Laccase activity. Rest of the samples showed activity of less than 10mm zone.

Carboxy Methyl Cellulose Agar (CMC) test for cellulose degradation by was carried out with all the isolates. Sample No.40 and 76 showed highest activity by showing a zone of 9.33mm followed by 104 and 112. Rest all showed less than 8.67mm. Sample No. 104 showed at 575nm, 1.83467 OD, giving enzyme activity of 0.19167 Mmol min−1 strongly supporting CMC agar medium test.

Lignin peroxidase enzyme activity was tested by Azure-B test. Highest activity was shown by sample no.59 and activity zone was measured 8.33mm followed by 86, 25, 41, 76 rest of the samples showed less activity /no activity. Sample No.86 showed at 651nm, 0.41 OD, giving Enzyme activity of 1.54 Mmol min−1 strongly supporting agar medium test.

Degradation and decolourization of RBBR dye test was carried out for all samples. Sample. No. 30 showed highest activity i.e., 7.00mm zone followed by no. 41, 59, 86, 40 and 46 whereas rest of the samples showed less and nil activity.

Weight of biomass was carried out for all species. Sample. No.123 recorded highest biomass weight (g) i.e., 0.91g followed by no.97, 93 and 106 whereas no.30 and 76 recorded lowest biomass weight 0.08 and 0.09 respectively.

Total Protein estimation of media was carried out for all samples. Sample No.104 showed a highest average OD value of 1.724 at 660nm, followed by no. 124 and 123 whereas lowest average OD value was found in no. 99 and 21 respectively. Highest protein activity was found in sample No.1 (0.301333333 Mmol min−1) whereas lowest activity was found in sample no. 104 (0.0853 Mmol min^{-1}).

Oxidation of guaiacol was monitored at 465nm by Manganese peroxidase activity.

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