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Evaluating ligninolytic enzymes from *Daedaleopsis confragosa* and *Phellinus pectinatus* for bio-pulping technique

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Abstract

In paper pulp industry it is needed to separate lignin from the cellulose for making white paper. Removal of lignin from wood involves use of different chemicals like sodium hydroxide (NaOH) and sodium sulfide (Na₂S) to remove noncellulose components and leaving the cellulose fibers intact. Thus chemical pulping involves lots of harsh chemical, which creates lots of water pollution and bio-pulping can be sustainably used to mitigate this issue.

In Bio- pulping is the process ligninolytic fungal enzymes (MnP, LiP, AAO, Lacasse) are used to break down lignin. Present work was aimed to analyse and characterize the lignocellulolytic enzyme activities of two white rot fungi *Daedaleopsis confragosa* and *Phellinus pectinatus*. *D. confragosa* produces ligninolytic enzymes like MnP, LiP, AAO and Laccase, *P. pectinatus* did not produce MnP and LiP, but produced AAO and Laccase. All the enzymes were stable at pH 5 and active till 45°C temperature. Results of the enzyme activity and stability to different pH and temperature, predicts the optimal conditions which would be suitable for its practical application in biodegradation processes, especially in pulp and paper industries for bio pulping.

Keywords: White rot fungi, ligninolytic enzymes, *Daedaleopsis confragosa* and *Phellinus pectinatus*, pH, Temperature

Introduction

Lignin is the second major reservoir of the fixed carbon sources in nature, comprising 15% of earth's biomass (Hammel 1992) [23]. The structural complexity of lignin requires different oxidative enzymes to be involved in its complete decay. Lignin peroxidase, Manganese peroxidase and laccase are the major lignin degrading enzymes (Niladevi 2009) [36, 30]. Other than this many other enzymes play role in the lignin degradation such as veraryl alcohol oxidase (Bourbonnains and Paice 1988) [6], Aryl alcohol dehydrogenase, Glyoxal oxidase (Kersten 1990) [28], Quinone oxidoreductase, aromatic acid reductase, vanillate hydroxylase, dioxygenase, catalase (Leisola and Fietcher 1985; Buswell and Eriksson 1988) [7] and aromatic aldehyde oxidase (Deobald and Crawford 1989) [53, 13].

Different ligninolytic enzymes have different mechanisms to degrade lignin. Action of ligninolytic enzymes is dependent on the generation of lignin free radicals which, because of their chemical instability undergoes many different cleavage reactions (Hemmel 1997) [24].

In concern to minimizing the environmental pollution problems, in industries like textile, paper etc. ligninolytic enzymes are used. Wesenberg *et al.* (2003) [49], Bettin Fernanda *et al.* (2019) [4] and Chander and Arora (2007) [9] studied the role of enzyme in textile dye decolorization and degradation of phenolic and non-phenolic aromatic compounds. Kirby *et al.* (2000) [29] reported that laccase from *Phlebia tremellosa* decolorized eight synthetic textile dye. Textile dye named Malachite green was successfully transformed by laccase from *G. lucidum* (Murugesan *et al.* 2009) [34]. Purified ligninolytic enzymes can also be used in paper pulp industry (Sahadevan LDM 2016) [45].

White rot fungi that produce ligninolytic enzyme are reported detoxification and decolorization of effluents of various industry like paper and pulp, coal conversion, petrochemical, alcohol distilleries, dyeing and textile industries (Rubilar *et al.* 2008; Raghukumar *et al.* 2008; D'souza *et al.* 2006) [23, 42, 14].

Ligninolytic enzymes are also used in denim washing (Pazarlioglu *et al.* 2005) [39], cosmetic industry (Aaslyng *et al.* 1996) [1], and biosensors

Materials and Methods

Enzymatic assays were performed in 100 ml of 3% liquid MEA medium. The medium was sterilized by autoclaving at 121 °C temperature and 15 psi pressure for 20 minutes. After cooling the medium, it was inoculated with 9 mm disc of 10 days old fungal culture in aseptic condition. The culture was incubated at 24±0.5°C for the desired incubation period. After completion of incubation period the cultures were taken out, mycelium broken with the glass pieces and then filtered through Whatman paper No. 1 disc. This filtrate was used as enzyme solution.

Fungal biomass

The concentration of biomass was quantified by dry weight analysis. Fungal biomass was harvested by filtration method using pre-weighted whatman paper no. 1 disc. Filter papers with the fungal biomass were dried in hot air oven at 70°C for 24 hrs and weighed using electronic balance (Chouhan and Nigam 2013) [10].

Evaluation of enzyme activities

I) Lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP activity): Lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) activities were measured according to the procedure of Castillo *et al.* (1994) [8] using substrate 0.167 mM 3-methyl-2-benzothiazolinone hydrazone (MBTH) which interact with 2.37 mM 3-(dimethylamino) benzoic acid (DMAB) producing a purple coloured reaction. Substrates were prepared on 0.1 M succinic-lactic acid buffer at pH 4.5. Cuvette (1.5 ml) contained 417.5 µl MBTH, 417.5 µl DMAB, 100 µl MnSO₄, 50 µl supernatant (enzyme) and 15 µl H₂O₂ to measure the enzyme activity. To distinguish between magnesium dependent and independent peroxidases reaction was performed in the presence and absence of 3mM MnSO₄. 4mM H₂O₂ was used to initialise the reaction. Reactions were carried out at 37°C and monitored spectrophotometrically at 590 nm during 1 min. The enzyme activity was calculated using as extinction coefficient $\epsilon = 53000 \text{ M}^{-1}\text{cm}^{-1}$.

II) AAO activity: Aryl alcohol oxidase (AAO) activity was estimated according to the procedure of Guillen *et al.* (1990) following the increase in absorbance due to the veratraldehyde formation from veratryl Aryl alcohol oxidase alcohol (3,4-dimethoxybenzyl alcohol). The reaction

mixture contained 5mM veratryl alcohol in a 0.1 mM sodium phosphate buffer pH 6 and 50 µl supernatant (enzyme solution). Reaction was performed at 30°C and visualized spectrophotometrically at 310 nm during 2.5 min and activity expressed in $\mu\text{kat/g dw}$ using $\epsilon = 9300 \text{ M}^{-1}\text{cm}^{-1}$ as the extinction coefficient of veratraldehyde.

III) Laccase activity: Laccase activity was determined by the oxidation of 2, 2'-azino-bis (3-ethylbenziazoline-6-sulphonic acid) (ABTS) by laccase causes by blue discoloration of the substrate (Niku-paavola *et al.* 1988) [35]. Enzyme assay were performed with 500 µM ABTS in 50 mM Sodium acetate buffer at pH 4.5. A 20 µl aliquot of enzyme solution was added to 580 µl of the ABTS. Change in the absorbance at 420 nm were observe ($\epsilon = 36,000 \text{ M}^{-1}\text{cm}^{-1}$).

Parameters affecting enzyme activity

I) Effect of pH: To estimate the effect of different pH on enzyme activity, all enzyme assays were carried out at different pH in 0.1 M of HCl-KCl buffer, pH 1 and 2; citrate buffer, pH 3 and 4; acetate buffer pH 5 and 6 and phosphate buffer, pH 7 and 8. Then all the enzyme assay were carried out spectrophotometrically (Trivedi 2002) [48].

II) Effect of Temperature: Enzyme containing supernatants were incubated at 25, 35, 45, 55, 65, 75 °C in water baths for four hours to allow ample time for denaturation. Then all the enzyme assay were carried out (Trivedi 2002) [48].

Statistical analysis

Data presented throughout this thesis were analyzed using Microsoft office excel. Results, where appropriate when subjected to an analysis of variance (ANOVA) testing at the $P=0.05$ level. Standard errors of the means (SE) were calculated.

Results

White rot fungi are known to produce different ligninolytic enzymes like MnP, LiP, AAO, Laccase. Screening of two white rot fungi was done to identify their ability of produce large quantities of ligninolytic enzymes

The activity of lignin degrading enzymes MnP, LiP, Laccase, AAO and fungal biomass was determined after an incubation period of 3-30 days. Biomass of both the fungi *D. confragosa* and *P. pectinatus* showed an increase from the third day of the incubation to 20 days period (0.12± 0.01 to 0.49±0.052) after which there was a reduction in the fungal biomass (Table 1 and 2).

Table 1: Bioassay of lignin degrading enzymes and fungal biomass of *Daedaleopsis confragosa*

Days	Fungal biomass (gm)	MnP (Eu/l/min)	LiP (Eu/l/min)	AAO (Eu/l/min)	Laccase (Eu/l/min)
3	0.12±0.01	0	0	13.32±0.39	0.63±0.01
5	0.13±0.005	0.062±0.001	0	15.49±0.24	0.74±0.01
10	0.17±0.01	0.39±0.036	0.94±0.17	19.53±0.45	0.87±0.005
15	0.35±0.02	0.77±0.036	1.71±0.072	20.51±0.24	0.91±0.023
20	0.49±0.052	0.94±0.06	1.77±0.07	22.41±0.47	0.97±0.01
25	0.4±0.01	1.7±0.09	2.49±0.03	24.25±0.17	1.07±0.03
30	0.25±0.02	0.63±0.10	1.8±0.07	23.43±0.23	0.87±0.027

Table 2: Bioassay of lignin degrading enzymes and fungal biomass of *Phellinus pectinatus*

Days	Fungal biomass (gm)	MnP (Eu/l/min)	LiP (Eu/l/min)	AAO (Eu/l/min)	Laccase (Eu/l/min)
3	0.12±0.008	0	0	19.50±0.45	0.69±0.02
5	0.13±0.005	0	0	22.87±0.30	0.86±0.005
10	0.14±0.002	0	0	26.67±0.36	0.92±0.002
15	0.16±0.005	0	0	27.27±0.51	0.93±0.01
20	0.21±0.01	0	0	28.30±0.70	1.07±0.02
25	0.16±0.007	0	0	29.49±0.29	1.21±0.008
30	0.16±0.016	0	0	28.72±0.28	0.93±0.004

The maximum growth of the fungus was observed on the 20th day (Fig. 1 A). Similarly the fungal biomass of *P. pectinatus* gradually increased in the fungal biomass till the 20th day, after that there was a decrease. On the 20th day fungal biomass was the highest (Table 2, Figure 1 B). In *D. confragosa* all the lignin degrading enzymes were produced in the maximum amount on 25th day (Table 1). Production of MnP was not initiated on 3rd day, but it slowly initiated by 5th day which increased and reached to a maximum on the 25th day, followed by which there was a decline (Fig. 2 A). LiP production initiated only on the 5th day and then similar to MnP the graph shows the peak activity on the 25th day after which it declines (Fig. 2 B). Enzymes AAO and Laccase were produced from day one with a gradual increase and reaching its peak on the 25th day and further declining the activity by 30th day (Fig. 2 C, D). Table 2 shows the results of ligninolytic enzyme activity of *P. pectinatus*. MnP and LiP enzymes were not produced by this fungus (Figure 2 A, B). Only AAO and Laccase enzymes were produced (Table 2). Similar to *D. confragosa*.

AAO and Laccase enzyme activity of *P. pectinatus* reached its peak on 25th day after which the activity declined (Figure 2 C, D).

Parameters affecting enzyme activity

Effect of pH: In the present study pH stability of the different ligninolytic enzymes (MnP, LiP, AAO and Laccase) have been evaluated. pH of the solution is very important for the enzyme activity. Table 3 shows pH stability of ligninolytic enzymes produced by *D. confragosa*. MnP is not found stable at very high acidic pH (1 to 4 pH), but it was stable in the pH range of 5-7 (Fig. 3 A). Similarly LiP was also not much stable in the initial pH range (1-3), but it was stable in the pH range of 4 to 7 (Fig. 3 B). As seen in figure 3 C AAO enzyme was stable in the pH range of 3 to 5. It was inactive in the neutral pH. Enzyme laccase showed its stability in the pH range of 4 to 6 (Figure 3 D). It was observed that all the enzymes showed less stability in basic pH 8 and optimum pH for all the enzymes was 5.

Table 3: Effect of pH on ligninolytic enzymes activity of *Daedaleopsis confragosa*

pH	MnP (Eu/l/min)	LiP (Eu/l/min)	AAO (Eu/l/min)	Laccase (Eu/l/min)
1	0.72±0.08	0.56±0.003	14.85±0.43	0.8±0.025
2	0.84±0.03	1.81±0.015	18.07±0.37	0.9±0.016
3	0.98±0.03	2.3±0.063	22.58±0.54	0.97±0.009
4	1.05±0.04	2.37±0.018	23.49±0.21	1.06±0.002
5	1.68±0.018	2.53±0.036	24.37±0.53	1.08±0.006
6	1.7±0.05	2.5±0.062	11.54±0.07	1±0.01
7	1.67±0.02	2.44±0.06	10.92±0.22	0.97±0.02
8	1.56±0.025	2.25±0.04	10.68±0.22	0.92±0.006

Table 4 shows effect of pH on different ligninolytic enzymes secreted by the *P. Pectinatus*. MnP and LiP enzymes were not produced by this fungus (Figure 3 A and B). AAO enzyme was stable in the pH range of 5 to 6. In highly acidic, neutral and in basic pH it showed reduction in

the enzyme activity (Figure 3 C). Laccase enzyme was stable in the pH range of 4 to 6 (Fig. 3 D). Similar to *D. confragosa* in *P. pectinatus* the optimum pH for all enzymes could be considered to be pH 5.

Table 4: Effect of pH on ligninolytic enzymes activity of *Phellinus pectinatus*

pH	MnP (Eu/l/min)	LiP (Eu/l/min)	AAO (Eu/l/min)	Laccase (Eu/l/min)
1	0	0	15.98±0.33	1.01±0.012
2	0	0	16.7±0.33	1.03±0.012
3	0	0	22.94±0.14	1.06±0.02
4	0	0	26.69±0.35	1.13±0.01
5	0	0	28.99±0.36	1.2±0.01
6	0	0	28.03±0.37	1.16±0.027
7	0	0	16.57±0.37	1.04±0.06
8	0	0	15.66±0.18	0.7±0.52

Effect of Temperature: Temperature is another important factor which determines the stability of the different ligninolytic enzymes (MnP, LiP, AAO and Laccase). The pH stability of different ligninolytic enzymes secreted by both the fungi were detected by incubating the enzyme

containing supernatants at 25, 35, 45, 55, 65, 75 °C in water baths for four hours to allow ample time for denaturation and then carry out the enzyme assays. Table 5 and 6 shows the effect of temperature on the ligninolytic enzyme activity of the two fungi.

Table 5: Effect of different temperatures on ligninolytic enzyme activity of *Daedaleopsis confragosa*

Temperatures (°C)	MnP (Eu/l/min)	LiP (Eu/l/min)	AAO (Eu/l/min)	Laccase (Eu/l/min)
5	1.69±0.07	2.65±0.2	24.04±0.18	1.05±0.004
15	1.72±0.042	2.57±0.16	24.73±0.25	1.05±0.004
25	1.73±0.031	2.49±0.036	24.26±0.18	1.08±0.036
35	1.69±0.034	2.34±0.13	24.05±0.2	1.01±0.01
45	1.69±0.007	2.18±0.03	24.61±0.2	1.04±0.005
55	1.109±0.036	2.01±0.063	23.42±0.27	0.93±0.005
65	0	0.96±0.03	18.35±0.33	0.73±0.004
75	0	0	0	0

Table 6: Effect of different temperatures on ligninolytic enzyme activity of *Phellinus pectinatus*

Temperatures (°C)	MnP (Eu/l/min)	LiP (Eu/l/min)	AAO (Eu/l/min)	Laccase (Eu/l/min)
5	0	0	29.47±0.31	1.18±0.021
15	0	0	29.09±0.35	1.22±0.013
25	0	0	29.49±0.29	1.22±0.008
35	0	0	29.63±0.09	1.21±0.016
45	0	0	28.17±0.33	1.17±0.006
55	0	0	25.34±0.83	1.1±0.02
65	0	0	19.14±0.37	0.86±0.005
75	0	0	0	0

Activity of MnP enzyme was stable in the temperature range of 5°C to 45°C produced by *D. confragosa*. At 55°C it showed slight decrease in the activity and at 65°C it abruptly decreased to zero (Figure 4 A). LiP enzyme was stable in the range of 5°C to 45°C after which the activity gradually declined, and at 75°C it became zero (Figure 4B). AAO activity was observed in the range of 5°C to 45°C and, at 55°C and 65°C activity reduced becoming zero at 75°C (Fig. 4 C). For laccase, 5°C to 45°C range was found suitable for its stability (Figure 4 D) and the activity becomes zero at 75°C. In this fungus all the enzymes showed the best stability at 25°C.

Table 6 shows the stability of different ligninolytic enzymes produced by *P. pectinatus* at different temperatures. AAO enzyme production was stable in the range of 5°C to 45°C and then it gradually reduced and became zero at 75°C (Fig. 4 C). Whereas laccase was found to be stable in the range of 5°C to 55°C and then reduced to zero at 75°C (Figure 4 D). At 25°C all the enzymes showed maximum activity.

Discussion

Enzymes were produced by these species indicating that the production of 1, 2 or 3 groups of ligninolytic enzymes also varied from species to species and strains of the same fungi. Elisahvili *et al.* (2009) [15] reported an absence of MnP production by few ligninolytic basidiomycetes fungi and production of these enzymes also differed with the strains. Of the two strains of *Fomesforment* (1BB9 and 1BB38) and three strains of *T. versicolor* (1BB5, 1BB13, 1BB16), *Fomesforment* 1BB38 and *T. versicolor* 1BB13, 1BB16 produce MnP but *Fomesforment* 1BB9 and *T. versicolor* 1BB5 do not produce MnP.

According to Pela'ez *et al.* (1995) [40] and Tekere *et al.* (2001) [47], in white rot fungi production of LiP is a rare. *Phellinus* sp. produced 0.044 U/ml laccase enzymes within 14 days of incubation and *P. igniarius* produced 0.044 U/ml laccase enzyme which agrees with our results for both the fungi. It also produced MnP (0.051 U/ml) and LiP (0.17 U/ml).

Pant and Adholeya (2007) [38] has studied ligninolytic enzyme activity of *Penicillium pinophilum*, *Alternaria gaisen* and *Pleurotus florida*. In this study they found that the laccase was produced by *P. florida* (0.806 U) within 10

days of incubation but in the present study same amount of the laccase was produced by *D. confragosa*, 25 days, of incubation with absence of MnP and LiP enzymes. MnP production of *P. pinophilum* is approximately same as that of *D. confragosa* but LiP production is much higher than the *D. confragosa*.

Studies on the lignin degrading enzyme Aryl-Alcohol Oxidase (AAO) and its enhancement are few (Ruiz-Duenas *et al.* 2006; Liers *et al.* 2011) [44, 31]. AAO is an FAD-containing extracellular enzyme (Guillen *et al.* 1992) [22]. Liers *et al.* (2011) [31] has found that *Phlebia radiata* (34 mU/g), *Bjerkandera adusta* (218 mU/g) and *Pleurotus eryngii* (13 mU/g), which agrees with AAO enzyme production in present study by both of fungi. Ruiz-Duenas *et al.* (2006) [44] has also found AAO enzyme activity using strain *Escherichia coli*.

Enzymes are very sensitive to the different factors (like cultivation condition, pH and temperature). In the present study we have studied the effect of pH on the activity of all ligninolytic enzymes. We have tested the stability of enzymes from pH 1 to 8.

AAO enzyme in *D. confragosa* was stable in the pH range of 3 to 5, while in *P. pectinatus* it was stable in the pH range of 5 to 6. Laccase produced by both the fungi showed maximum activity in the same pH range. For both the fungi pH 5 was found to be optimal for stability of all ligninolytic enzymes.

The optimum pH for laccase activity has been found to vary from one substrate to another when compared with the data obtained from other sources. The ligninolytic enzyme activity of purified laccase and MnP from *Pleurotus ostreatus* were found to be completely stable in large pH range (4-8) with an optimum range at pH value of 4 (Aslam and Ashger 2011) [3]. Xin and Geng, 2011 evaluated the effect of different parameters on the production of laccase by *Trametes versicolor*, and observed that the optimum yield of laccase (range of pH 5-9) at initial pH value of 7 was 1.2 fold of that obtained at initial pH 4.

Gianfreda *et al.* (1999) [19] earlier reported laccase from *Trametes versicolor* to be three times more active with DMP than 2, 2'-azino-bis (3-ethylbenziazoline-6-sulphonic acid) diammonium salt. Also Nyanhongo *et al.* (2002) [37] reports laccase activity by a strain of *Trametes modesta* to be

optimum at pH 4 using 2, 6 dimethoxyphenol (DMP) as the substrate. In the present study with ABTS as substrate both fungi *D. confragosa* and *P. pectinatus* produced an optimal laccase enzyme production and activity at pH range 3-5 with maximum at pH.

Michniewicz *et al.* (2006) [32], observed that the pH optima for the laccase enzyme production by *Cerrena unicolor* to be high in the acidic range (pH 2.5 to 3) with the ABTS as the substrate.

LiP enzyme activity was stable in the pH range of 3 to 5 produced by the fungus *Phanerochaete chrysosporium* (Bosco *et al.* 2010) [5], which is in accordance with the results of the *D. confragosa*.

After the pH one of the major factor affecting the stability of the fungus is temperature. Optimum temperature varies with the variation in the strain (Farnet *et al.* 2000) [16]. During the pulping process the pulp is exposed to the high temperature and at high temperature the enzymes become denatured and inactive. So the aim of this study was chalk the ability of fungus to produce the ligninolytic enzymes which remain stable at a higher temperature.

In the present study all ligninolytic enzymes become inactive at the temperature 75°C except MnP produced by the *D. confragosa*, which became inactive at 65°C. 25°C was found to be the optimum temperature for the stability of all the enzymes. The stability remained continuous and constant till 45°C and after that it started showing decrease in the enzyme activity.

Hossain and Anatharaman (2006) [27] reported a similar observation in the ligninolytic enzyme activity of *Trametes versicolor*. For many enzymes denaturation of proteins begins to occur at 45 to 50°C temperatures. Sensitivity of a protein to denaturation at elevated temperatures can vary widely with medium pH and the influence of various

temperatures, pH combinations may differ tremendously from enzyme to enzyme (Shuler and Kargi 1992; Pelczar *et al.* 1993) [46, 47]. In the present study all the ligninolytic enzymes optimally increased at a pH value of 5 and temperature 25°C with the stability remaining continuous till 45°C.

Farnet *et al.* (2000) [16] reported laccase produced by *Marasmius quercophilus* to be were also stable at 60°C. Monterio *et al.* (1998) [33] could use the fungal laccase in the pre bleaching stages at 55°C successfully.

Laccase produced by the white rot fungus *Cerrena unicolor* increased with an increase in the temperature and is highest at 60°C and after which it suddenly declined with increase in temperature (Michniewicz *et al.* 2006) [32]. In the present study laccase produced by both the fungi *D. confragosa* and *P. pectinatus* remained stable till only 55°C.

Using a strain of *Phanerochaete chrysosporium* Bosco *et al.* (2010) [5], studied the effect of temperature on the stability of the LiP enzyme. They observed that LiP produced by *P. chrysosporium* was stable at 40°C. Lip produced by *D. confragosa* was found to be stable till 55°C. which indicates that the temperature stability of LiP enzyme produced by the different fungal species can differ from each other.

Aslam and Asgher (2011) [3], conducted an experiment to evaluate temperature stability of laccase and MnP produced by the *Pleurotus ostreatus* and they found that till 55°C both the enzyme remained active and after that their activity showed reduction a result completely in accordance with the present study.

Laccase enzyme produced by most of the fungi were shown to be stable below 50°C (Yaropolov *et al.* 1994; Wood 1980) [52, 50]. *D. confragosa* and *P. pectinatus* produce thermostable enzymes so both are the suitable strains for being used in paper pulp industry.

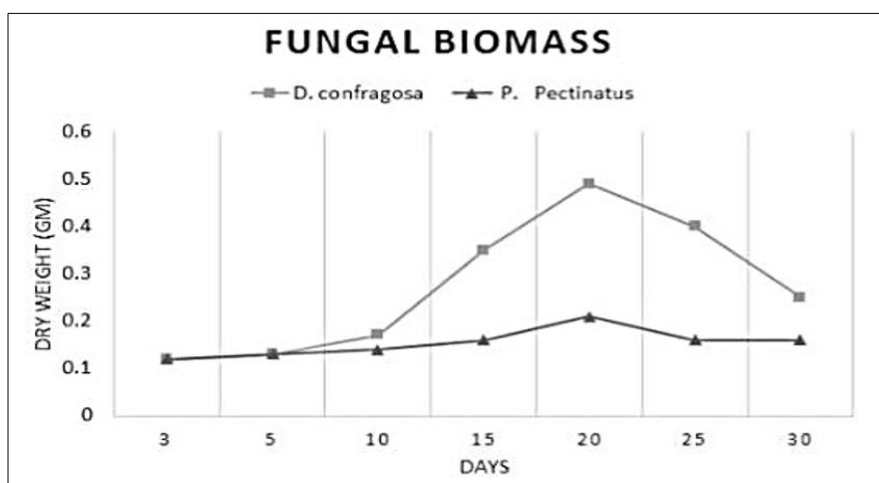
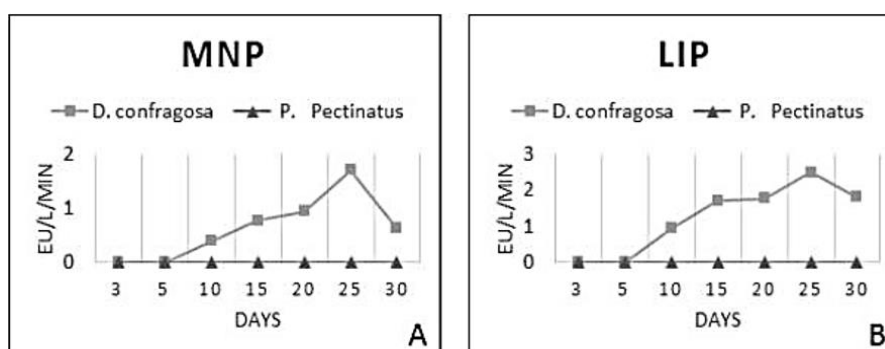


Fig 1: Variation in fungal biomass with difference incubation period



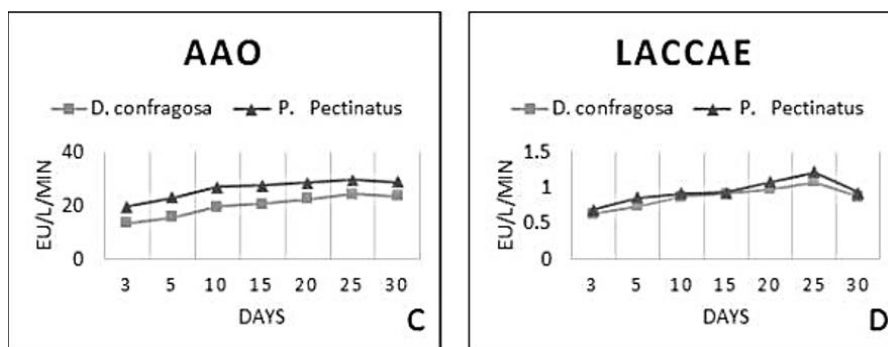


Fig 2: Ligninolytic enzymes produced during different incubation period

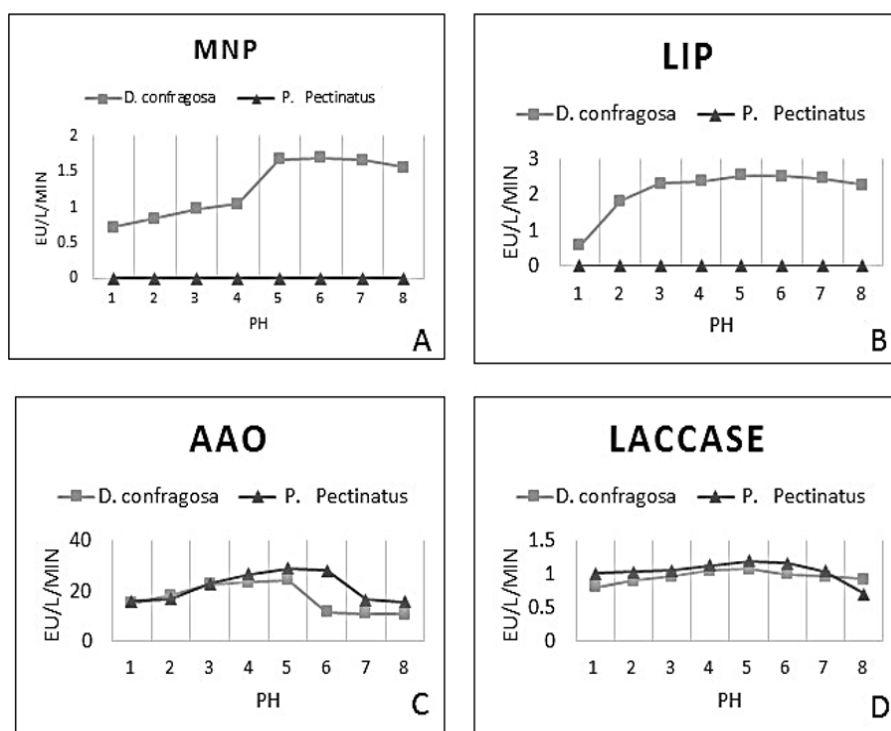
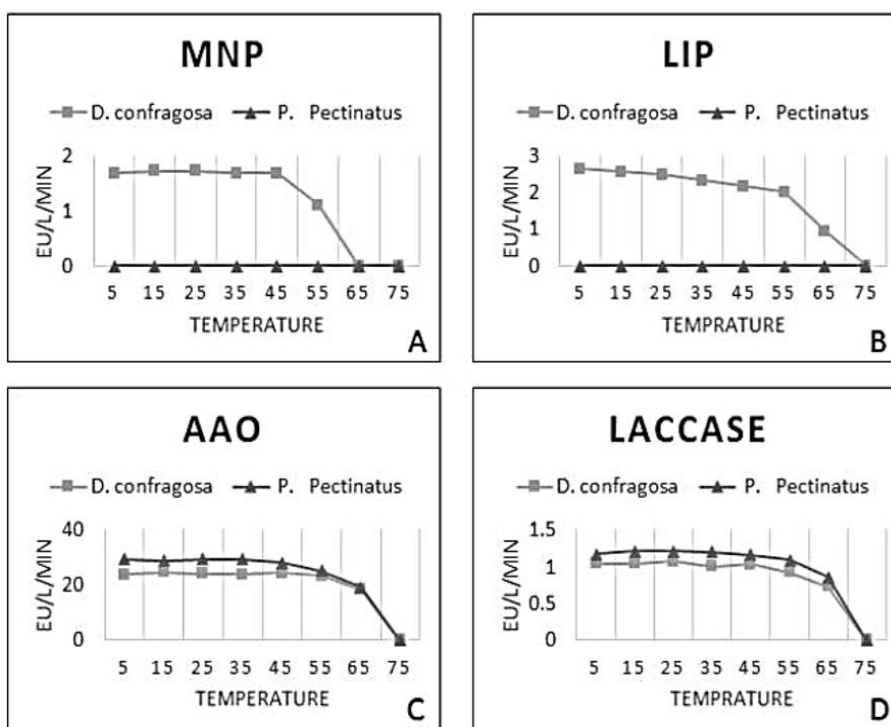


Fig 3: Effect pH on ligninolytic enzyme activity



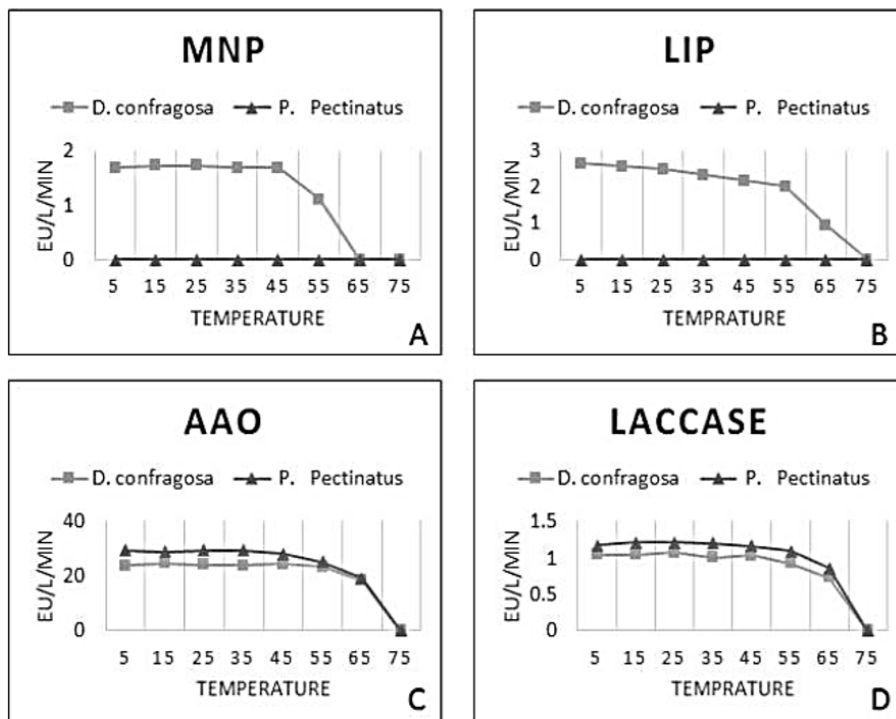


Fig 4: Effect of different temperatures ligninolytic enzyme activity

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