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RESEARCH ARTICLE

EVALUATION OF WHITE ROT FUNGI FOR THE PRODUCTION OF LIGNIN MODIFIED ENZYMES COLLECTED FROM GOA FOREST AREAS IN WESTERN GHATS, INDIA

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ABSTRACT

White rot fungi is commonly known as wood decaying fungi which grows on moist wood and digests converts it to rot. Rice straw degradation by white rot fungi has potential to increase the digestibility of animal feed and its nutritional value for the production of lignolytic enzymes. Twenty white rot fungal isolates were obtained from wood samples. These fungal isolates were subjected to qualitative screening tests for lignolytic activity on solid media containing several polymeric dyes like poly R-478, Tannic acid, Azure-B, Remazol brilliant blue R. Out of twenty isolates four isolates with appreciable enzyme activity were selected based on the dye decolourisation property. The optimized conditions resulted in high lignin modified enzymes (LME) production with the 745 Uml^{-1} of Laccase, 265 Uml^{-1} of MnPase and 142 Uml^{-1} of Peroxidase within 12 days of incubation, at the pH - 6.5 and 35˚C of temperature. Purification studies were conducted by ammonium sulphate precipitation followed by SDS PAGE analysis in which the molecular weight was determined as 45 kD, 57 kD then HPLC analysis reveals peak at 19 mins at specific wavelength of A_{280}. The present findings revealed the the strain LMCD-2 is an efficient organism for lignolytic enzyme production.

INTRODUCTION

Lignin is the major structural component of woody plants which gives structural rigidity to cell wall. Lignin is a poly hydrocarbon aromatic compound. Due to its complex structure lignin is very difficult to hydrolyze so it can be persistent in environment as undegradable compound. Lignin degradation is important in biotechnological industries worldwide as degraded lignin releases cellulosic chains, which have specific industrial applications. Many studies have indicated that most of the microorganisms, especially white rot fungi, have the ability to degrade lignin due to their extracellular enzyme production ability. Laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) are three types of lignin-degrading enzymes that have been implicated in the lignin biodegradation (Perez and Jeffries, 1990; Pointing, 1999). For screening of potent lignin modified enzymes (LME) producing fungi several dyes were used. The assumption is the decolourisation of dye is due to destruction of chromophore structure, will happen only with release of lignin modified enzymes. The present study focused on production of lignin modified enzymes with selected white rot fungal species isolated form forest areas of Mollem in Goa, Western Ghats. Western Ghats are the eighth hottest hotspots of biodiversity of the world where very rare species of plants and animals population were discovered. Lignin modified enzymes (LME) activity was confirmed by qualitative screening methods and production was carried out by using chemical defined medium the cultural conditions were optimized to acheive high lignin modified enzymes (LME) activity. The solid state fermentation (SSF) is an attractive process to produce fungal microbial enzymes using lignocellulosic materials from agricultural waste due to its lower capital investments and lower operating cost. The effect of pH, temperature, initial glucose concentration, on enzyme production was analyzed. The optimization yields maximized enzyme production. The purification studies were carried out for characterization of enzymes.

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MATERIALS AND METHODS

Isolation of lignolytic fungi

White rot fungus was isolated from the rotten wood barks, collected from the different locations of Mollem forests in Western Ghats. Collected samples were brought to laboratory and cut in to small pieces, washed thoroughly by rinsing in sterile water for 20mins and surface sterilization was done by soaking in 75% alcohol for 1min followed by 10% sodium hypochloride and 95% ethanol for 30 sec. Samples were blotted between two folds of sterilized filter paper and were placed on 2% malt extract agar with chloramphenicol to inhibit growth of bacteria. The plates were incubated at 25°C. Hyphal tips of colonies growing out from the plant tissue were transferred to fresh potato dextrose agar slants. The fungal stock cultures were maintained through periodic transfer on Potato Dextrose agar slants at 4°C until use. The isolated strains were carefully identified by morphological characteristics include colour of the colony and growth pattern studies, as well as their vegetative and reproductive structures observed under the microscope.

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. The potent strains were selected based on the characteristic observation and dye decolourisation.

Poly R-478 decolourization

A single agar disc (5 mm diam.) of the actively growing fungus was taken from the leading edge and inoculated on lignin-modifying enzyme basal (LMB) medium containing (per litre) 20g glucose, 1g KH_2PO_4, 0.5g $C_4H_{12}N_2O_6$, 0.5g $MgSO_4.7H_2O$, 0.01g $CaCl_2.2H_2O$, 0.01g yeast extract, 0.001g $CuSO_4.5H_20$, 0.001g $Fe_2(SO4)_3$, and 0.001g $MnSO_4.H_2O$ and incubated for 10 days in dark. The polymeric dye Poly R-478 (0.02g) was used as a poly aromatic lignolytic indicator and the pH was adjusted to 5.5 (*Pointing, 1999*). During incubation, mycelial growth and decolouration of Poly R-478 was observed. The isolates decolourising the polymeric dye were selected for further study.

Tannic acid agar test

To detect lignin modified enzymes (LME) production, the fungal isolates were grown on solid Czepek-Dox medium incorporated with 2ml of 20% glucose and 1ml of 1% tannic acid. The plates were observed for browning of the media due to polyphenoloxidase activity.

Azure –B test

LBM medium was prepared supplemented with 0.01 % w/v Azure B dye and 1.6 % w/v agar and autoclaved. Aseptically 1 ml of a separately sterilized 20 % w/v aqueous glucose solution was added to each 100 ml of growth medium prepared. The isolated fungi were aseptically transferred to Petri dishes. The plates were incubated at 25^{o}C in darkness and examined the plates for 10 days at 24 hours interval. Recorded the production of lignin peroxidase and Mn dependent peroxidase as clearance of blue colored medium.

Remazol brilliant blue R decolourization

peroxidase activity of fungal isolates was performed using the dye Remazol brilliant blue R (RBBR). The functional output was measured based on the amount of dye decolonization on agar plates. The purified cultures of fungal strains derived from stock cultures were inoculated on PDA agar plates and incubated at 25°C. After 48 hr, 5 mm agar disks were acquired from the growing margins of test strains and these were inoculated onto a screening medium agar plate and incubated at 25 °C for 15 days. The diameter of clear zones were measured and recorded. The control plate was not inoculated with test fungus. The fungal isolates which are positive for the screening methods were considered as potent lignolytic strains and further used for enzyme production.

Quantitative estimation of lignin- degrading enzymes

Kirk's medium was used as fermentation media for quantitative estimation of enzyme activities from the selected strains (Rigas *et al*., 2005)

Sample preparation

Wheat straw and rice straw were collected and used as substrates. The straw materials were dried at 50^{o}C and finely powdered for SmF and Chopped into pieces for SSF.

Inoculum preparation

The inoculums was prepared by transferring two agar discs from seven days old culture grown on solid agar media into 250-ml flasks containing 50ml malt extract broth media and incubated at 25ºC under stationary conditions for 6 days. After 6 days of growth, the cultures were homogenized and further used as inoculum for the estimation of lignolytic activity.

Submerged Fermentation

Out of twenty species isolated four of the most promising lignolytic fungi LMCD-1to LMCD-4 were grown on agar plates and the agar discs were transferred to a 250 ml Erlenmeyer flask containing lignin-modified enzyme basal medium (LBM), which include 2g/L lignocellulosic powder. The LB medium without lignin powder was used as control. The flasks were incubated in rotary shaker at 25^{o}C for 4-14 days.

Solid State Fermentation

Solid State Fermentation was carried out at 27^{o}C in 250ml conical flasks containing 4g of lignocellulosic substrate moistened with 12ml of LB broth medium. 3 ml of homogenized mycelium was used to inoculate the flasks. After 4-14 days of incubation the extra cellular enzymes were extracted on a mechanical extractor two times with 25ml of distilled water. The solids were separated by filtration through cheese cloth followed by centrifugation at 6000 x g for 15 min at 4^{o}C.

Enzyme assays

After incubation the samples were collected from the inoculated flasks and the solids were separated by filtration through cheese cloth followed by centrifugation at 6000xg for 15 min at 4^{o}C. The culture broth was sampled for enzyme activity. The filtrate was tested for enzymes activity as follows. (Mtui and Masalu, *2008*).

Laccase activity (Lac)

Laccase activity was measured by monitoring the oxidation of 1mM 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 50mM Na-acetate buffer and measured at 420nm (Eggert *et al.*, 1996). One unit of laccase activity was defined as the amount of enzyme that oxidize 1µ mol ABTS in 1 min.

Lignin peroxidase activity (LiP)

LiP activity was measured by monitoring the oxidation of veratryl alcohol in the presence of H_2O_2 at 310nm. One unit of LiP activity was defined as the amount of enzyme catalizing the formation of 1 µ mol of veratraldehyde per minute (Takamiya *et al.*, 2008).

Manganese peroxidase activity (MnP)

MnPase activity was measured by using guaiacol as a substrate. The increase in absorbance at 465nm due to oxidation of guaiacol was measured. One unit of MnP activity was defined as activity of an enzyme that catalize the conversion of 1µ mole of guaiacol per minute.

Protein estimation

The protein content of the culture filtrate was estimated according to the method of Lowry *et al*., (1951) using bovine serum albumin (BSA) as a standard curve.

Optimization of production of Lignin modified enzymes (LME)

The Lignin modified enzymes (LME) production was optimized using submerged fermentation. For the initial optimization of the medium, the traditional method of "one variable at a time approach" (OFTA) was used by changing one component at a time while keeping the others at their original level. The selected lignololytic strains were grown in selected media consisting of selected substrates for enzyme production. Studies were performed in shake flasks to optimize different fermentation conditions for hyper Lignin modified enzymes (LME) production.

Effect of initial pH and temperature on enzyme production

The effect of initial pH was tested by inoculating the selected isolate in LB liquid medium, which was adjusted to pH 3.0-8.0 using 1M HCl or 1M NaOH. The optimum temperature for enzyme production was investigated by incubating samples of the selected isolates in LB broth

at 20°C, 30°C, 37°C, 45°C and 60°C). The flasks were incubated in darkness at 120 rpm on a rotary shaker for 12 days. The culture broth was separated from the mycelium by centrifugation at 10000 rpm for 10 minutes and the supernatant was assayed for enzyme activities.

Effect of varying time

Lignin modified enzymes (LME) activity was measured at regular intervals while fermentation was observed at 29±1˚C for a period of 4 to14 days and maximum enzyme production was determined on 7^{th} day of submerged fermentation (SmF) and 12^{th} day of solid state fermentations(SSF) respectively.

Partial purification

The mycelial extract of potent strains of lignin modified enzyme property was passed through whatman no.1 filter paper to remove fungal mycelia, after which the filtrate was combined with ammonium sulfate to 80% saturation then incubated for 60 min at room temperature with gentle stirring. The precipitate formed was collected by filtration and dissolved in 20mM 1-methyl piperadine buffer, pH 4.5. After centrifugation (15,000 × *g*, 15 min) the enzyme solution was dialyzed against the same buffer at 4^{O}C for 12 hours. The column had been equilibrated with 20mM1-methyl piperadine buffer, pH 4.5, after which the enzyme was eluted with a linear gradient of 0–1.0M NaCl in the above buffer at the flow rate of 5 ml/min. Laccase-active fractions of 10 ml were collected, combined, dialyzed overnight against 50mM phosphate buffer, pH 7.0 at 4 ◦C, then concentrated with an ultra filtration membrane. The partially purified laccase obtained was stored at 4 ◦C and used for subsequent characterization.

Gel Electrophoresis

The SDS- PAGE was performed using the method of *Laemmli et al., 1970*. The stacking and separating gel concentrations were 5% and 12% of polyacrylamide, respectively. After the electrophoresis, the gels were stained with Coomassie brilliant blue R-250 (Sigma) for visualization of protein bands.

HPLC analysis

For lignin modified enzymes (LME) analysis samples of 20 fold concentrated extracellular fluid were dialyzed against 10mM sodium acetate, pH 6.0. They were then filtered through filters. HPLC analysis was performed on a Gilson system equipped with a Holochrome detector using a Pharmacia FPLC mono Q ion exchange column. The mobile phase consisted of a gradient from 10mM to 1M sodium acetate, pH6.0, and was applied over a 40 min period at flow rate of 2ml min^{-1} with constant monitoring at 280 nm.

RESULTS

About twenty white rot fungal sp. isolated from collected samples (Figure 1 and 2).

Fig. 1. Isolation spots of white rot fungi (tree barks with fungal association)

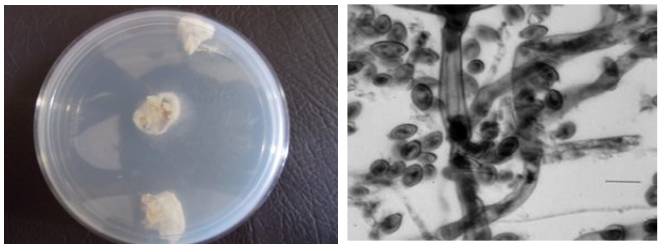
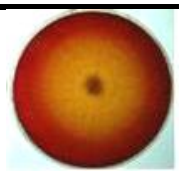
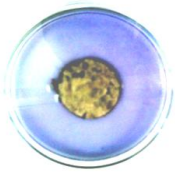


Fig. 2. *In vitro* Culturing and microscopic observation of white rot fungi showing hyphae and conidia

Table 1. Qualitative Screening for lignin degrading fungi for enzyme production

Screening test	Results obtained	Characteristic observation
Poly R-478 decolourisation	Four strains were more effective in Poly R-decolourisation and the colonies appear more distinct, being formation of clear yellow hydrolysis zone after incubation.	 Poly R-478 decolorizing activity
Tannic acid agar test	Four strains showed brown colored oxidizing zone around the colonies were selected as positive for lignin modified enzymes (LME) production	 Positive strain showing the browning of the medium
Azure –B test	Four strains were positive for Azure-B dye decolourisation confirms the production of lignin-peroxidases, Manganèse oxidases	 Azure –B decolorization
Remazol brilliant blue R decolourisation	Four strains were positive for Remazol brilliant blue R dye decolourisation confirms the production of lignin-peroxidases.	 Remazol brilliant blue R Decolourisation

Out of 20 fungal sp., 4 isolates having lignolytic properties with appreciable enzyme activity were selected based on the several screening tests. The isolated strains were carefully identified by morphological characteristics include color of the colony and growth pattern studies, as well as their vegetative and reproductive structures observed under the microscope identified as white rot fungi. The four strains which were passed all the qualitative screening tests were selected for further studies and they were named as LMCD-1, 2, 3 and 4 respectively. The fungal sp, were inoculated into LB medium consisting of various lignocellulosic substrates in the conical flasks for both SmF and SSF. Maximum enzyme production was determined for the selection of a specific substrate for specific strain. In both submerged fermentation and solid state fermentation appreciable enzyme activity was obtained for LMCD-2 and LMCD-3. LMCD-2 accumulated high enzyme activity in presence of rice straw as ligninocellulosic substrate as compared with remaining isolates (Table 2 and 3). Rice straw was selected as best substrate for further studies as the results shows efficient enzyme activity with rice straw as compared to wheat straw. The appreciable enzyme activity was obtained for LMCD-2 strain followed by LMCD-3 strain. Uml^{-1} of Laccase, 265 Uml^{-1} of MnPase, and 142 Uml^{-1} of Peroxidase where as LMCD-3 secreted 146 Uml^{-1} of laccase and 98 Uml^{-1} of MnPase and 68 Uml^{-1} of Peroxidase respectively. LMCD-2 was selected for further study due to its high lignin modified enzymes (LME) activity.

Submerged fermentation

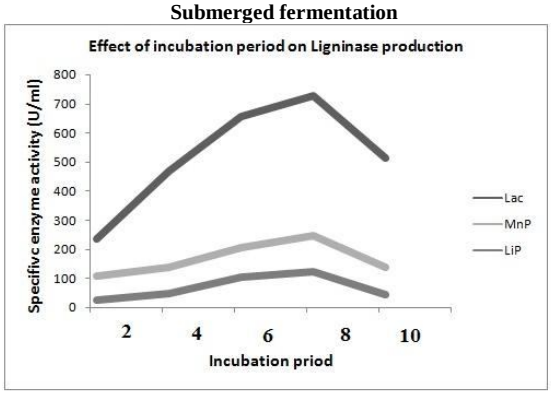


Table 2. Lignolytic enzyme activity of white rot fungi under optimized conditions of submerged fermentation

S.No.	Isolate	Substrate	Laccase (Uml^{-1})	MnP (Uml^{-1})	Peroxidase (Uml^{-1})	Final pH	Protein content (mg/ml)
1.	LMCD 1	Wheat straw	46	0	traces	4.5	0.143
		Rice straw	92	0	traces	4.1	0.164
2.	LMCD 2	Wheat straw	507	172	83	4.0	0.202
		Rice straw	631	202	119	3.9	0.225
3.	LMCD 3	Wheat straw	67	55	23	3.9	0.194
		Rice straw	119	81	46	3.9	0.172
4.	LMCD 4	Wheat straw	34	traces	0	4.6	0.098
		Rice straw	50	traces	traces	4.2	0.121

Table 3. Lignolytic enzyme activity of white rot fungi under optimized conditions of solid state fermentation

S.No.	Isolate	Substrate	Laccase (Uml^{-1})	MnP(Uml^{-1})	Peroxidase(Uml^{-1})	Final pH	Protein content (mg/ml)
1.	LMCD 1	Wheat straw	53	traces	0	3.9	0.147
		Rice straw	112	traces	traces	4.6	0.178
2.	LMCD 2	Wheat straw	598	127	82	4.2	0.225
		Rice straw	670	263	131	3.9	0.239
3.	LMCD 3	Wheat straw	83	68	33	4.1	0.205
		Rice straw	123	96	68	4.0	0.198
4.	LMCD 4	Wheat straw	34	0	0	3.9	0.116
		Rice straw	60	traces	traces	4.1	0.145

Table 4. Comparision of increased production of lignin modified enzymes (LME)s before and after optimisation

Isolate	Initial lignolytic activity			Optimised Lignolytic activity		
	Solid state fermentation					
	laccase Uml-1	MnP Uml-1	LiP Uml-1	laccase Uml-1	MnP Uml-1	LiP Uml-1
LMCD-2	670	263	131	745	265	142
LMCD-3	123	96	68	146	109	85
	Submerged fermentation					
LMCD-2	631	202	119	720	229	126
LMCD-3	119	81	46	138	98	52

Optimization of production of Lignin modified enzymes (LME)

Lignin modified enzymes (LME) activity was measured at regular intervals at 29±1˚C for a period of 12 days and the period of maximum enzyme production was determined on 7^{th} day of submerged fermentation and 12^{th} day of solid state fermentations respectively. (Figure 3). The optimal pH range for Lignin modified enzymes (LME) production by LMCD-2 in LB medium was 4.0-6.5 at 30°C. The highest activity was achieved at pH 6.5 and the activity decreased considerably when the pH of the medium was higher than 6.5. Enzyme production by LMCD-2 at various temperatures following 5 days of incubation was studied. while maximum enzyme activity was achieved at 30°C as compared to the temperature 37°C and 45°C. The enzyme production was highest with LMCD-2, 3 at 30 ˚C temperature with the pH-6.5 and 12 days of incubation period in presence of rice straw as substrates. (Figure 4, 5). A greater ligninolytic activity was observed in LB media supplemented with lignocellulosic substrate, (rice straw) than in media supplemented with Poly R-478. LMCD-2 secreted 745

Solid state fermentation

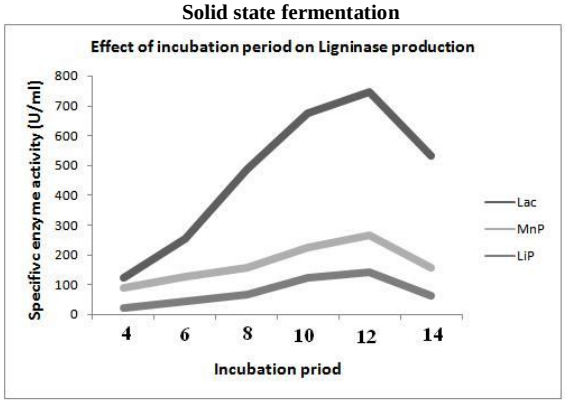
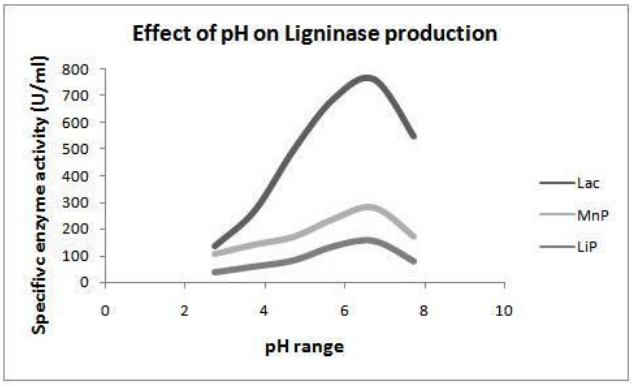


Fig.3: Effect of incubation period on Lignin modified enzymes (LME) production

Submerged fermentation



Solid state fermentation

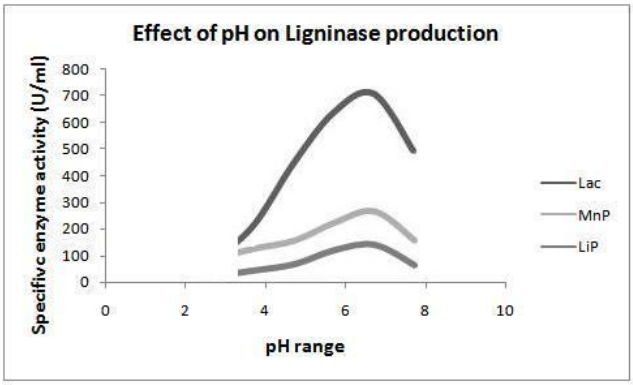
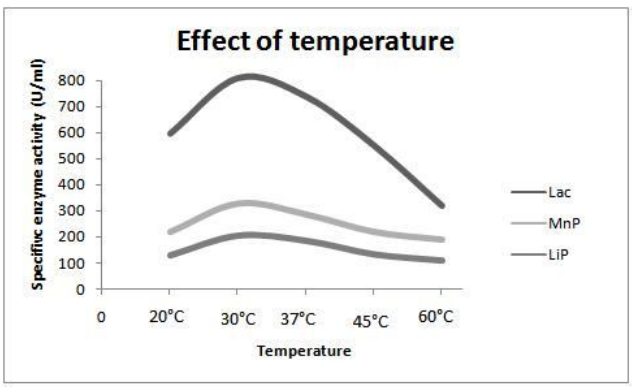


Fig. 4. Effect of pH on Lignin modified enzymes (LME) production

Submerged fermentation



Solid state fermentation

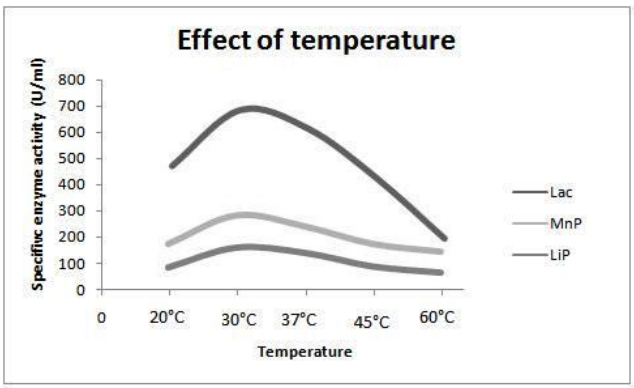


Fig. 5. Effect of temperature on Lignin modified enzymes (LME) production

Gel Electrophoresis

Zymogram of partially purified lignin modified enzymes were obtained by SDS- PAGE. Comparing with the molecular mass of protein marker bands by staining with Coomassie brilliant blue R-250 (Sigma) the molecular weight of lignin modified enzymes (LME) bands were determined. As per the zymogram the molecular weight of lignin modified enzymes (LME) was determined as 24 kDa, 45 kDa and 57kDa. **(**Figure 6)

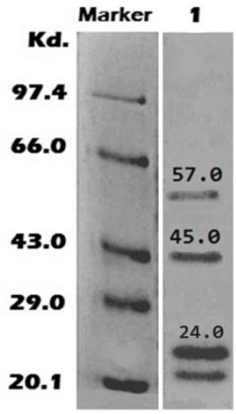


Fig. 6. Sodium Dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) of lignin modified enzymes (LME)

HPLC analysis

Lignin modified enzymes (LME) enzyme HPLC analysis was performed on a Gilson system. The mobile phase consisted of a gradient from 10mM to 1M sodium acetate, pH6.0, and was applied over a 40 min period at flow rate of 2ml min^{-1} with constant monitoring at 280 nm. The enzymatic reaction was studied for 24h. The results for 24h of reaction between veratryl alcohol and enzymes recovered in the concentrate, after ultra filtration of lignases produced by white rot fungi. All things considered, the results from HPLC analysis confirm the existence of lignases in the enzyme mixture produced by white rot fungi that act synergistically to release veratryl oxidised products with lignin modified enzymes (LME)s. The retention time for oxidised veratryl alcohol is 19 mins. (Figure 7)

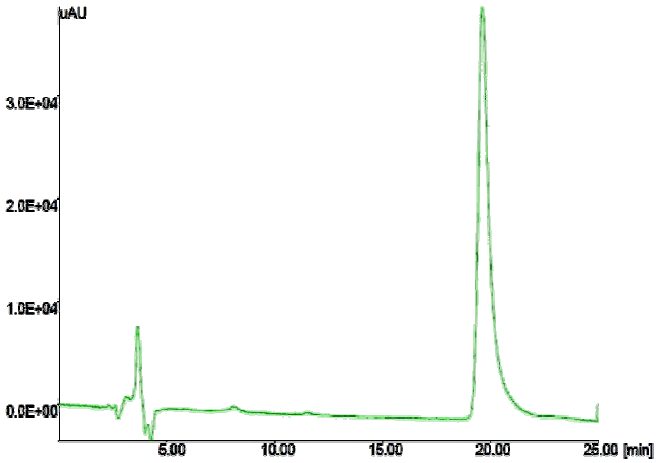


Fig. 7. HPLC chromatogram between veratryl alcohol oxidizing activity (0.4mM) and the enzyme mixture showing elution times of 4 mins and 19 mins at specific wavelength A_{280}. Mobile phase consisted of a gradient from 10mM to 1M sodium acetate, pH6.0, and was applied over a 40 min period at flow rate of 2ml min^{-1}

DISCUSSION

Lignin is a complex chemical compound most commonly occurs in plant cellwall thickenings especially in tracheids, sclereids and xylem. The term lignin was introduced in 1819 by de Candolle which means wood. White rot fungi has the ability to degrade a broad spectrum of structurally diverse aromatic compounds like several environmental pollutants (Field *et al.,* 1993). The lignin degradation by white rot fungi is a non-specific mode. The present work reveals that the optimised conditions for excessive lignin modified enzymes (LME) production by selected white rot fungi. White rot fungi commonly grows on the trunks of woody plants and convert the lignocellulosic substances in to rot by oxidizing the lignin content present in plant cell wall thickenings. Lignin modified enzymes (LME) production depends upon the composition of the fermentation medium. The isolated working cultures were grown on a defined medium as decribed previously (Collins and Debson, 1995). Medium optimization for over production of the enzyme is an important step and involves a number of physico-chemical parameters such as the incubation period, pH, temperature, carbon and nitrogenous sources (Eggert.C *et al*., 1996). The decolourisation property simply conveys the oxidising property of chromophores present in polymeric dyes which were chosen due to its simplicity and reliability as previously demonstrated. Our results were consistent with the results obtsined by Pointing, 1999; Zheng *et al., 1999;* Couto *et al.,* 2000; Leung and Pointing, 200. Decolorization of the dye Azure-B by isolated fungi has been positively correlated with production of lignin peroxidase and Mn dependent peroxidase (Archibald, 1992). We found increased enzyme activity when the cultures were grown in presence of a rice and wheat straw as a lignocellulosic carbon source. We also observed in the process that presence of an inducer like tween 80 and veratryl alcohol but it was found that it is not required to boost the production as there is no any characteristic increased results were obtained (Couto *et al*., 2000). Some authors have suggested that the increase in enzyme production in presence of veratryl alcohol as it has the ability to protect the enzyme from hydrogen peroxide dependent inactivation (Tonon and Odier, 1988). In purification method we determined if the mode of purification process would improve the enzyme recovery and activity and it found increased enzyme activity. This may be due to the crude purification method which may have resulted in diminishing the enzyme recovery and potency. With better and sophisticated methods of purification we may be able to get good recovery of the enzyme without letting its activity get affected.

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REFERENCES

Collins, P.J and Debson, A.D.W. (1995) Biotechnol. Lett. 17, 989-992.

Eggert.C, U. Temp, and K-E. Eriksson, (1996). The ligninolytic sistem of the white rot fungus Pycnoporus cinnabarinus: purification and characterization of the laccase. Applied and Environmental Microbiology 62: 1151-1158.

Field, J.A., E. Dejong, G. Feijoo-Costa and deBont J.A.M.(1993) Trends Biotechnol. 1, 44-49.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. and Randal, R.J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem., 193, 262.

Mtui, G. and Masalu, R. (2008). Extracellular enzymes from brown-rot fungus Laetiporus sulphureus isolated from mangrove forests of coastal Tanzania. Academic Journals, 3 (4), 154-161.

Perez, J. and Jeffries, T.W. (1990). Mineralization of 14c-ring-Iabeled synthetic lignin correlates with the production of lignin peroxidase, not of manganese peroxidase or laccase. Applied and Environmental Microbiology 56: 1806-1812.

Pointing, S.B. (1999). Qualitative methods for the determination of lignocellulolytic enzyme production by tropical fungi. Fungal Diversity 2: 17-33.

Rigas, F.; Dritsa, V.; Marchant, R.; Papadopoulou, K.; Avramides, E.J.; and Hatzianestis, I. (2005). Biodegradation of lindane by Pleurotus ostreatus via central composite design. Environment International, 31, 191–196.

Takamiya, M.; Magan, N. and Warner, P.J. (2008). Impact assessment of bisphenol A on lignin-modifying enzymes by basidiomycete Trametes versicolor. Journal of Hazardous Materials, 154, 33–37

Toca-Herrera J. L, Osma J. F., and Rodríguez Couto S., Potential of solid-state fermentation for laccase production, Communicating Current Research and Educational Topics and Trends in Applied Microbiology, 391-400.

Zeng, *et al*. (1999). Enhancement of interdecadal climate variability in the sahel by vegetation interaction, science 286, 1537.

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