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# Induction of lignolytic enzyme activities in different agro residues by the white rot fungi, *Pleurotus sajar-caju*

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**Abstract**
The white rot fungi, *Pleurotus sajar-caju* secretes the major lignin modifying enzymes Laccase (Lac) and Manganese peroxidase (MnP) with low levels of Lignin peroxidase (LiP) during their secondary metabolism that is capable of mineralizing lignin. Assay of enzymes under liquid screening test indicated significant levels of laccase (0.56 U/ml), LiP (0.054U/ml) and MnP (0.21 U/ml) by *P. sajar-caju*. Induction of lignolytic enzymes by *P. sajar-caju* was tested in different farm agro wastes *viz*., sugarcane, banana, millets, pulses, cocopeat, cocoa wastes, coir pith and oil palm under solid state fermentation. Among the enzymes, laccase and MnP was produced in significant levels by *P. sajar-caju* from 21$^{st}$ to 28$^{th}$ day under solid state fermentation (SSF). Among the substrates tested, significant levels of laccase was secreted in residues of oil palm, banana, followed by sugarcane and pulses where as maximum MnP was produced residues of oil palm, banana, sugarcane, cotton and pulses. LiP though produced was in trace amounts which showed preference over the substrates like sugarcane banana, pulses and oilseeds. Substitution of malt extract medium with organic substrates *viz.*, wheat bran and ground nut cake each @ 5 per cent and inorganic substrates *viz*., copper sulphate and manganese sulphate each at 150 µM enhanced the lignolytic enzymes of *P. sajar-caju* in liquid medium. The results from this study offers scope for application of white rot fungi *P. sajar-caju* for biodegradation of agrowastes as well for recycling of the agro wastes for the commercial production of edible mushrooms under Integrated farming system to benefit the farming community as well to tap industrially important lignolytic enzymes like laccases.

**Keywords:** Agro wastes, Biodegradation, manganese peroxidase, laccase, lignin peroxidase, *P. sajar-caju*

## 1. Introduction
The white rot fungi are an interesting group of fungi belonging to basidiomycetes and capable of degrading the complex aromatic biopolymer lignin using their natural lignolytic machinery. Lignolytic enzymes include laccases (Lac), lignin peroxidises (LnP), managanese peroxidise (MnP), versatile peroxidase (VP), Aryl alcohol oxidases, glycol oxidases are well known for their potential applications in conversion of variety of agrowastes, bioremediation of soil and many industrial applications. But, however two or more enzymes may be produced simultaneously by one type of white rot fungi; but their secretion is in less quantity which ultimately depend on the type of substrate, pH of the substrate, temperature and cultivation conditions. The agro wastes produced from various crops pose a lot of environmental pollution especially the coir pith, sugarcane wastes and banana plant wastes contain high lignin content and are left unused or burnt *in situ* that creates environmental pollution. In that way the lignolytic machinery of the white rot fungi can be exploited for biodegradation of the agrowastes in a simpler manner these agrowastes can be used to convert in to organic manure using the white rot fungi.
Industrial importance of the model white rot fungi, *Phanerochaete chrysosporium* has been studied with reference to the laccase and manganese peroxidase mediated bioconversions [1, 11, 31]. Different substrates like pulse husk, paddy straw, rice bran, saw dust, sesame oil cake and wheat bran were used for induction of cellulase and laccase production, where wheat bran enhanced cellulase production whereas paddy straw and sesame oil cake enhanced laccase production of *P. sajar-caju* under solid-state fermentation [22]. Among the oyster mushroom species, *P. sajar-caju* also has the capabilities to utilize the lignocellulosic paddy straw for conversion in to protein rich edible mushroom in many sub tropical countries where rice is

cultivated as major food crop. Apart from these scientists are now focussing research on the identification of best suitable substrates for colonisation by the white rot fungi *Pleutotus* sp. for bioconversion in to food. In this context, the enzyme secretion pattern by *P. sajar-caju* in different agrowastes under solid state fermentation is studied with a view to identify the best suited substrate that will support the colonisation and utilization of agrowaste both for mushroom production as well to convert in to biomanure. Also, the production of lignolytic enzymes is influenced by wide variety of phenolic and aromatic substances [17]. Also the increased production of laccases in white rot fungi grown on cotton stalk [1], wheat bran [25] and barley bran [9] has been reported. Certainly it is expected that the best substrate can still be efficiently utilised by substituting with organic and inorganic amendments that can stimulate the enzyme secretion activities.

## 2. Materials and Methods

The white rot fungal culture *Pleurotus sajar-caju* (Gen bank accession number JN 849390.1) was used for the studies. The fungi was multiplied in Malt extract medium in Petri dishes and used for further studies.

### 2.1 Liquid screening test for lignolytic enzymes

Screening of the white rot fungi *P. sajar-caju* for the production of major lignolytic enzymes *viz*., laccase (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP) were performed in liquid medium. For this purpose, the culture media was prepared as per the method [24, 10]. The media was inoculated with the 9 mm mycelial disc of *P. sajar-caju* and incubated for 7-10 days. The culture filtrate was centrifuged at 10,000 rpm for 30 min at 4 °C and assayed for the enzyme activity. The laccase activity was assayed using 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as the substrate [23]. The laccase reaction mixture contained 0.5 ml of 0.45 mM ABTS, 1.2 ml of 0.1 M phosphate buffer (pH 6.0) and 0.5 ml of enzyme sample [29]. The oxidation of the substrate (ABTS) was read at absorbance of 420 nm, molar extinction co-efficient of $3.6 \times 10^4$ $cm^{-1}$ $M^{-1}$). The assay for the lignin peroxidase activity was estimated using method of [16] with Pyrogallol as substrate. The assay mixture contained 1 ml of enzyme sample, 0.2 ml of 0.1 M pyrogallol, 2 ml 0.1 M phosphate buffer pH 6.5 with 0.1 ml, 0.1 M $H_2O_2$ [20]. The enzyme activity was determined at 30 sec intervals for 5 min at 436 nm using molar extinction co-efficient of 2470 $M^{-1}$ $cm^{-1}$. The manganese peroxidase (MnP) activity was assayed as per the method [31] with sodium malonate as substrate. The assay mixture contained 1 ml of enzyme sample, 0.2 ml of 1mM $MnSO_4$ in 0.05 M sodium malonate, 0.2 ml of 0.1mM $H_2O_2$. The oxidation of sodium malonate was read at absorbance of 270 nm ($E_{270}$= 11.59 $mM^{-1}$ $cm^{-1}$). One unit (U) of enzyme activity is defined as the amount of enzyme which produced 1 mM of product/min/ml.

### 2.2 Solid screening test for laccase production.

Based on the results obtained from liquid screening, the laccase enzyme secretion of *P. sajar-caju* was identified in solid medium with the indicator compound guaiacol as substrate. A special medium with the following composition (g/l): 3.0 peptone, 10.0 glucose, 0.6 $KH_2PO_4$, 0.001 $ZnSO_4$, 0.4 $K_2HPO_4$, 0.0005, $FeSO_4$, 0.05 $MnSO_4$, 0.5 $MgSO_4$, 20.0 agar (pH 6.0) supplemented with 0.02% guaiacol was used [7]. A 9 mm mycelial disc of pure culture of *P. sajar-caju* was placed in the centre of the medium in the Petri plate and incubated at room temperature for 5-7 days. Laccase activity was visualized as formation of reddish brown zones in the medium due to oxidative polymerization of guaiacol by the laccase enzyme secreted by *P. sajar-caju*. Lignolytic basal medium (LBM) was supplemented with 0.1% w/v ABTS and inoculated with test fungus and examined for 10 days [21]. Production of laccase was recorded with the formation of a green color in the growth medium due to the oxidation of ABTS to ABTS-azine in the presence of laccase.

### 2.3 Lignolytic enzyme production by *P. sajar-caju* in different agrowastes under solid state fermentation (SSF)

The solid state bioconversion or fermentation (SSF) of agro wastes *viz*., coir pith waste, farm wastes of millets, banana leaves, pulses, oilseeds, cotton stalks, sugarcane trashes, cocoa leaf wastes, oil palm fibre wastes and spent coco peat by *P. sajar-caju* were carried out in 250 ml conical flasks. About 20 g of each agro wastes (chopped) was placed in individual flasks and 60 ml of distilled water was added to give a moisture content of 70%. The flasks were plugged with cotton and autoclaved at 121 °C for 15 min. The 9 mm mycelial disc of *P. sajar-caju* was inoculated into the individual flask containing the agrowastes and incubated at 30 °C for 35 to 40 days. Three replications were maintained. Samples (1g) were drawn at 7 days intervals up to 35 days from each flask and analyzed for laccase, lignin peroxidase and Manganese peroxidase enzyme activities as described earlier. The experiment was replicated thrice.

### 2.4 Enhancement of lignolytic enzyme production of *P. sajar-caju* by substitution with amendments under *in vitro* condition in liquid medium

Organic amendments *viz*., wheat bran, rice bran, maize flour, groundnut cake powder and horse gram powder were supplemented in malt extract liquid medium at different concentrations (1, 5 and 10%) to record the induction of enhanced lignolytic enzymes. The inorganic amendments *viz*., copper sulphate and manganese sulphate at the rate of 50 µM, 100 µM, 150 µM and 200 µM were added to the malt extract liquid medium in 50 ml flasks. A 9 mm mycelial disc of *P. sajar-caju* was inoculated and incubated at 30 °C for 5-7 days. The culture filtrate was centrifuged at 10,000 rpm for 30 min at 4 °C and assayed for the enhanced production of laccase, LiP and MnP enzyme activity as described earlier in liquid screening test. Treatment with only malt extract served as control. The experiment was replicated thrice.

## 3. Results and discussion

The lignolytic enzymes are secreted during their secondary metabolism and are non specific with high oxidative capacity that is capable of mineralising lignin. Lignin degradation not only involves mediators, but also the enzymes with synergistic action. However the secretion of lignolytic enzymes depends on the genetic variability of organisms, pH, moisture and type of substrates. Quantitative assays (liquid screening) are normally performed for screening of white rot fungi for their lignocellulolytic enzymes spectrophotometrically, whereas qualitative assays are performed in solid and liquid media impregnated with indicators and the results are correlated based on visual observation of the colour reactions. It is considered that quantitative analysis provides accurate information on the amount of enzyme secreted, but qualitative test also provides further confirmation of the secreted enzymes through visual observation.

### 3.1 Secretion of lignolytic enzymes under liquid and solid medium

Enzyme secretion in liquid medium test showed significant levels of laccase (0.56 U/ml), LiP (0.054U/ml) and MnP (0.21 U/ml) by *P. sajar-caju*. Results showed that laccase was the major enzyme secreted by followed by MnP and LiP. Further, laccase production by *P. sajar-caju* was confirmed by solid screening test using guaiacol as substrate with reddish brown zones in the entire plate which indicated the oxidation of guaiacol (Plate 1). Also with (ABTS) 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as substrate, development of intense of green color indicated the oxidation of 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) to ABTS-azine in the presence of laccase (Plate 2).

### 3.2 Lignolytic enzyme production *P. sajar-caju* in different agro wastes and amendments

Solid state fermentation method especially utilizing agrowastes for production of laccases finds application in biotechnological processes in industries for bioconversions as it's closely resembles the natural conditions for the uniform fungal colonization at low moisture conditions. In our study, *P. sajar-caju* produced the lignolytic enzymes *viz*., laccase, Lignin peroxidase and Manganese peroxidase in all the substrates under solid state fermentation (SSF). However, level of each enzyme production varied between substrates. Also the enzyme production increased from 7$^{th}$ day till 28$^{th}$ day with peak enzyme production on 28$^{th}$ day. After 28 days, the lignolytic enzyme production decreased in all the substrates.

The enzyme assay performed on 28$^{th}$ day revealed significant levels of laccase production was recorded in oil palm fibre waste (0.83 U/ml) and in banana residues (0.65 U/ml), cotton stalks (0.64) followed by sugarcane and pulses (0.56 and 0.53 U/ml respectively). Also laccase production was observed in residues of oilseeds, coir pith, cocopeat and cocoa with reduced level (Fig. 1). It is clear that though all the substrates supported laccase production by *P. sajar-caju,* mycelial colonisation of was well observed from 10 days itself in residues of oilpalm and banana. MnP was produced in all the substrates under SSF. However, significant levels of MnP was produced in oil palm (0.186 U/ml) and banana (0.168 U/ml) followed by sugarcane, cotton, pulses sugarcane and millets (0.145 to 0.158 U/ml). Very low levels of LiP was produced in oil palm wastes, sugarcane trash, followed by residues of pulses, banana leaf, cocoa leaf wastes and oilseeds (0.035 to 0.081 U/ ml respectively).

Reports show that *P. sajar-caju* produced copious amounts of laccase and low levels of MnP and two veratryl alcohol oxidases [4]. The white rot fungi, *Pleurotus pulmonarius* produced significant levels of laccase in orange wastes with maximal activity of 12.2 U/mL obtained after 20 days of cultivation [14]. Also the agroresidues of banana, oil palm, coir pith and millets supported laccase and maximum MnP was produced in sugarcane, pulses, cotton and millets where as trace levels of LiP was secreted in substrates like sugarcane banana, pulses and cotton [27]. From the results, it is clear that laccase is the major lignolytic enzyme followed by MnP and LiP involved in *P. sajar-caju* for degradation of agro wastes (Fig. 2&3).

The lignolytic enzyme production in white rot fungi is highly influenced by nutrients such as nitrogen, copper and manganese [2]. The results from the present study showed that malt extract substituted with the organic substrates *viz*., wheat bran, rice bran, maize flour, groundnut cake powder and horse gram powder and inorganic substrates *viz*., Copper sulphate and Manganese sulphate at different concentrations induced lignolytic enzyme production by *P. sajar-caju* in liquid medium. In our study, substitution of organic amendments *viz*., wheat bran, rice bran, maize flour, groundnut cake and horse gram powder in malt extract medium at different concentrations were found to enhance the major lignolytic enzymes. Addition of wheat bran and ground nut cake each at 5% enhanced the production of laccase (0.67 and 0.63 U respectively), LiP (0.18 and 0.22 U respectively) and MnP (0.13 and 0.17 U respectively). The reports supported our work that supplementing the wheat bran solid medium with 2 mmol/litre of copper sulphate allowed induction of higher levels of laccase enzyme by the white rot fungi, *Fomes fomentarius* [18]. It has been discussed by various authors that wheat bran contains soluble phenolic compounds like ferulic acid, coumaric acid and syringic acid which stimulates laccase enzyme secretion in white rot fungi [11,12].

The inorganic amendment Copper sulphate at 150 µM enhanced laccase (0.59U), Manganese sulphate at 100 µM enhanced the production of LiP and MnP (0.29 and 0.34 U respectively) (Fig. 2). Though the control treatment supported good mycelial growth, the levels of laccase, LiP and MnP produced was less. Similarly, the inorganic substrates, copper sulphate at 150 µM concentration contributed for enhanced levels of laccase and MnP (0.57 U/ml and 0.37 U/ml respectively) and copper sulphate at 100 µM enhanced the LiP (0.28U/ml). Also, Manganese sulphate at 150 µM enhanced the laccase (0.19U/ml), LiP (0.49U/ml) and MnP (0.50U/ml) enzyme levels compared to other concentrations and control. Mn2+ performs the role of mediator for MnP, where MnP catalyzes the oxidation of Mn2+ to Mn3+ that forms complex with oxalate and other chelators that enhance the activity of MnP [26].

The phenolic inducers like Pentaflurophenol enhanced the laccase enzyme production in *P. flabellatus* in liquid medium [28]. In our study, rice bran did not enhance the laccase enzyme level but highest production of laccase in rice bran supplemented medium was recorded in *Coriolous versicolor* [6]. Earlier reports showed that addition of manganese promoted the production of MnP in *Pleutotus ostreatus* [15] and *P. pulmonarius* [5]. Several authors explained that the regulation of laccase isoforms by copper ions in *Trametes versicolor*, *T. pubescens* and *T. versicolor* and Manganese ions in *P. ostreatus* occurs at gene transcription level [8, 13, 19]. However, copper sulphate and manganese sulphate at higher concentration beyond 150 ug reduced the enzyme production by *P. sajar-caju.*

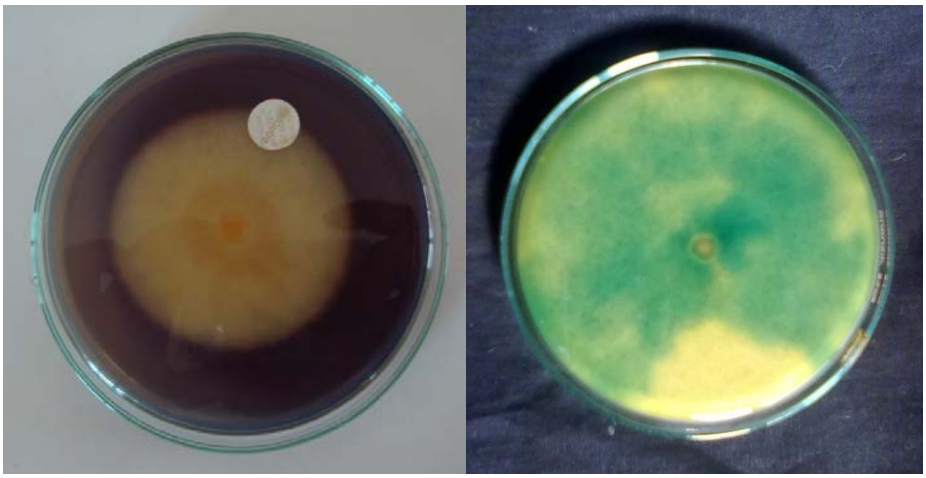
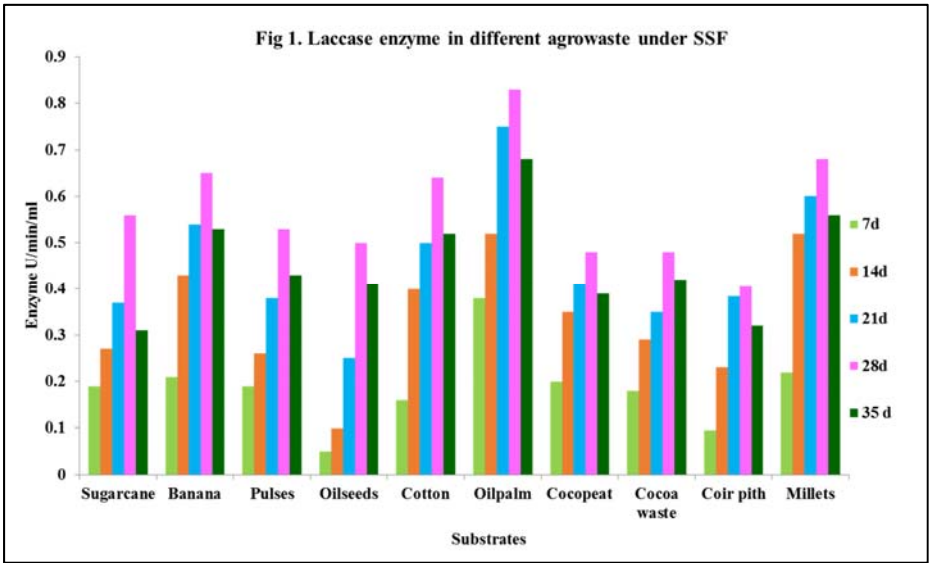
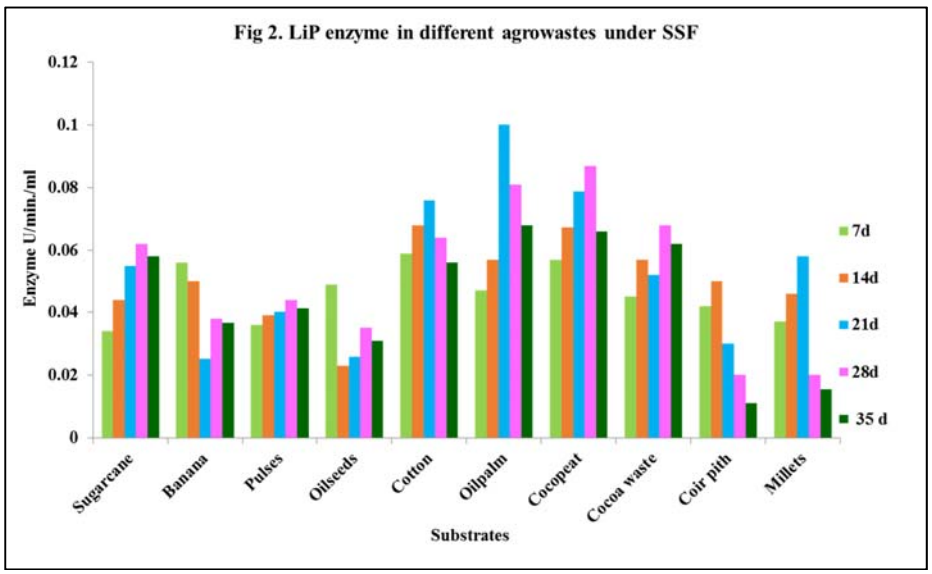
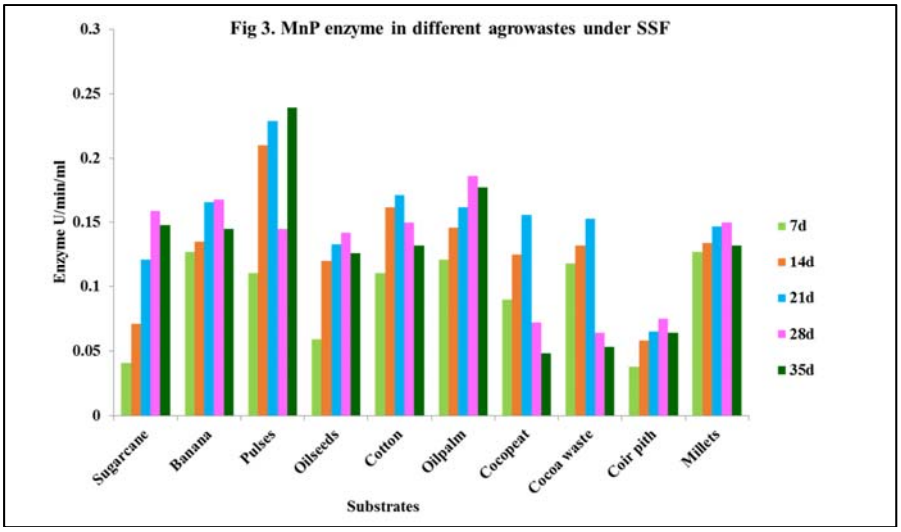


**Plate 1:** Laccase enzyme in Guaiacol **Plate 2:** Laccase enzyme in ABTS






## 4. Conclusion

As discussed, the agrowastes like sugarcane trash, coir pith, coco peat, millets and oilseeds wastes are left unused or burnt *in situ*. Based on the above studies, the white rot fungi like *P. sajar-caju* can be used both for bioconversion of agrowastes for production of edible mushroom as well to degrade the left over agrowastes in to organic manure. Through the process of bioconversion of agroresidues in to rich organic manure that can be applied back to the soil will certainly minimise the application of inorganic fertilizers. More over such enriched soil facilitates harbouring of natural antagonistic microorganisms that in turn leads to good crop stand of better quality. Also during the process of biodegradation addition of amendments *viz.,* wheat bran and ground nut cake each at 5% concentration and copper sulphate and Manganese sulphate each at 150 µM to enhance the activity of lignolytic enzymes that can certainly enhance the degradation potential of white rot fungi for bioconversion of agro wastes in to edible mushroom production and also to degrade high lignin containing agroresidues in to nutrient rich organic manure that can best fit under integrated farming system. The best suited agroresidues can be selected to tap large quantities of lignolytic enzymes especially laccases that can find application in paper and pulp industry for bioleaching and biopulping processes.

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