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Original article

Extracellular secretion of lignocellulolytic enzymes by diverse white rot basidiomycetes fungi

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Abstract

Rich source of biota includes several white rot fungi, were reported in Western Ghats regions of Tamil Nadu, India. The effective identification of white rot fungi and their lignocellulolytic enzymes play a crucial role for degradation of agricultural and industrial wastes to maintain sustainable ecosystem. Thus, the present study was conducted to assess the secretion of lignocellulolytic enzymes of diverse white rot fungi, collected from Western Ghats regions of Tamil Nadu, comprising of Kotagiri and Ooty of the Nilgiri district, Siruvani and Anaikati of Coimbatore district. A total of 16 white rot fungi belonging to 6 genera, *viz*., *Pleurotus* spp., *Lentinus* spp., *Ganoderma* spp., *Trametes* spp., *Pycnoporus* spp. and *Schizophyllum* spp. were collected and identified through morphological and molecular methods. The activity of cellulase enzyme was prominently visible in *Pleurotus pulmonarius, P. sajar-caju, Pycnoporus sanguineus, P. ostreatus, Ganoderma gibbosum* and *Schizophyllum commune* and hemicellulolytic activity was maximum in *Trametes ijubarskii, Lentinus crinitus, P. sanguineus* and *G. gibbosum* . The quantitative and qualitative screening for major lignolytic enzymes, *viz*., laccase (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP) revealed significant levels of Lac secretion when compared to LiP and MnP by *P. pulmonarius, P. sajar-caju* and *Schizophyllum commune*. The syringaldazine well test further confirmed the secretion of laccase as major enzyme followed by LiP and MnP. Also the Lac and LiP activity was maximum at pH of 4.5 and MnP activity at pH of 5.0 by *P. pulmonarius, P. sajar caju* and *S. commune*. Temperature of 30°C induced maximum secretion of Lac, LiP and MnP by these three fungi. A significant level of Lac was observed on 4th day after inoculation while LiP and MnP was maximum on 7th and 8th day for *P. pulmonarius* and *P. sajar-caju*. Whereas, *S. commune* secreted significant levels of Lac on 10th to 11th day, while LiP and MnP on 6th and 7th day. This study paves the way for use of *P. pulmonarius, P. sajar-caju* and *S. commune* for biodegradation and bioconversion of agrowastes.

Key words: Basidiomycetes, laccase (Lac), lignin peroxidase (LiP), manganese peroxidase (MnP), white rot fungi

1. Introduction

Microorganisms, in general are capable of degrading and utilizing cellulose, hemicelluloses and lignin biopolymer as carbon and energy sources. However, only a smaller group of sporocarpic fungi known as white rot fungi possess the unique ability to efficiently degrade lignin to CO_2. The most rapid degraders in this group are basidiomycetes (Sanchez, 2009) that secrete greater amounts of lignocellulolytic enzymes and bring about the degradation of complex macromolecules such as cellulose, hemicellulose, chitin, lignin, lipid and protein in the substrates (Narsi *et al*., 2006; Kuforiji and Fasidi, 2008). Wood-rotting basidiomycotina fungi are usually divided into white-rot, brown-rot and litter decomposing fungi. White rot basidiomycetes fungi grow in clusters on dead wood throughout the year. Most of the white rot fungi are wood rotting basidiomycetes, are capable of decaying lignin based polymers and several variable recalcitrant environmental pollutants due to its inherent ability to secrete cellulolytic and lignolytic enzymes such as lignin peroxidase, manganese peroxidase and laccases which aid the decaying process of dyes and inorganic pollutants (Ogbo *et al*., 2006). Lignin peroxidase, polyphenol oxidase and laccases produced by white rot fungi, degrade many aromatic compounds (Singh and Pakshirajan, 2010).

Lignin biodegradation by white rot fungi is an oxidative process mediated by a unique set of extracellular enzyme system known as ligninolytic system. It is comprised of three major classes of enzymes designated as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) (Ryu *et al*., 2003; Couto, 2009; Patrick *et al*., 2010). These enzymes also find application in pulp bleaching, degradation of recalcitrant organo-pollutants such as polyaromatic hydrocarbons, chlorophenols and polychlorinated biphenyls in food industry, cosmetology and in pharma industries (Martinez *et al*., 2005; Maciel *et al*., 2010). Among the white rot fungi, *Phanerochaete chrysosporium* is the best studied lignin degrading white rot fungus that produces distinctive set of lignolytic

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enzymes for degradation of lignin in natural lignocellulosic substrates and various xenobiotic compounds including dyes (Wesenberg *et al*., 2003). Similarly, *Phlebia radiata, P. ochraceofulva, Dichomitus squalens, Rigidoporus lignosus* and *Junghuhnia separabilima* are efficient producer of LiP, MnP and laccase enzymes (Hatakka, 1994). Qualitative assays are powerful tools used in screening fungi for lignocellulose degrading enzyme production (Ponting, 1999). Lignin peroxidase (LiP) and manganese peroxidase (MnP) are commonly known glycosylated heme protein peroxidases produced by white rot fungi that degrade complex materials in the atmosphere (Young and Akhtar, 1998). However, the enzyme secretion is not substrate specific but rather dependent on the potential or capability of the white rot fungal species and its diversity, as the white-rot fungi that produce MnP (but not LiP) can also degrade nonphenolic lignin structures.

Basidiomycetes species are considered as a very interesting group of fungi, owing to their ability to accommodate even detrimental conditions of the environment where they continue to act as natural lignocellulose destroyers. Currently scientists are focusing on the studies related to quantification of these enzymes in diverse white rot fungi on lignocellulose bioconversion that could be useful for identifying the potential species or strain that could find place in bioconversion of agroresidues for improving the cultivation technology of unexploited potential mushrooms, in biotechnological processes such as biopulping, kraft bleaching and bioremediation of industrial effluents and soil. Recently, lignin degrading enzymes are used for degradation of pharmaceuticals and their related materials from surface, ground and drinking water (Wen *et al.*, 2009) and as a component of enzyme cocktail in deinking of paper fiber. In order to assess the factors which are responsible for the decomposition or degradation of plant materials, consisting of lignin or cellulose, play a essential role for maintenance of biological balance of environment. The present study aims at studying the major lignolytic enzyme pattern of diverse white rot fungi through qualitative and quantitative assays in order to screen to potential white rot fungi. The emanating results will lead to strain improvement of existing ones, optimizing the fermentation factors for tapping more enzymes to find out a strain that can be used for various agricultural and industrial applications.

2. Materials and Methods

2.1 Chemicals

2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), pyrogallol, guaiacol and syringaldazine were purchased from Sigma Aldrich. All the other reagents, chemicals, solvents and media were purchased from HiMedia, Mumbai, India. All chemicals used in this work were of analytical grade.

2.2 Collection and isolation of white rot fungi

The white rot basidiomycetes fungal sporocarps and rotten wood containing fungal propagules were collected from Western Ghats regions adjoining areas of Tamil Nadu, comprising of Kotagiri, Nilgiris, Siruvani and Anaikati. The fungal propagules were isolated from degraded wood materials, using malt extract agar medium. The plates were incubated at room temperature (25 ± 2°C) for 7-10 days to ensure growth of fungi. The fully grown fungal cultures were stored and used for further studies.

2.3 Morphological and molecular characterization of white rot fungi

Morphological characters of collected mushrooms were denoted by referring to monographs (Hall *et al*., 2003; Kaul, 1997; Nonis, 1981; Russel, 2006) and mushroom identification keys provided by ICAR-Directorate of Mushroom Research, Solan, Himachal Pradesh, India. All the 16 isolates were grown in malt extract broth and the mycelial mat was collected from each and ground with lysis buffer for DNA extraction and isolated DNA was subjected to PCR amplification as per the method (White *et al*., 1990). The actual size of amplified products was identified based on molecular weight marker, using 2% agarose gel with electrophoresis and product was sent to Chromos Biotech Pvt. Ltd., Bengaluru, India for sequencing. The sequence of the partial 18s rRNA were compared with deposited entries available in the National Centre for Biotechnology Information (NCBI) database, using basic local alignment search tool (BLAST) algorithm. Based on the database comparative statement, nucleotide sequences of white rot fungi were deposited in GenBank, NCBI.

2.4 Screening of white rot fungi for major extracellular lignolytic enzyme production

2.4.1 Quantitative screening of the white rot fungi under liquid medium

Sixteen white rot fungal cultures were grown in special culture media, prepared as per the method of Sayadi and Ellouz (1995). The laccase activity was assayed using 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrate (Rasera *et al*., 2009). 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 (Vaidyanathan *et al*., 2011). The oxidation of the substrate (ABTS) was read at absorbance of 420 nm, molar extinction co-efficient of 3.6×10^4 cm^{-1} M^{-1}. Lignin peroxidase assay was estimated using method of Kang *et al*. (1993) 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. 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 of Wariishi *et al*. (1992) 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. Based on the results obtained through quantitative screening, those promising white rot fungal cultures, *viz*., *P. pulmonaris, P. sajar caju* and *S. commune* found to secrete significant levels of Lac, LiP and MnP were subjected to further conformation through qualitative screening.

2.4.2 Qualitative screening of the white rot fungi under solid medium

All the sixteen white rot fungal cultures were screened for the production of cellulase, hemicellulase, laccase (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP) activity by solid screening as per Ponting (1999). The lignolytic enzymes produced by the fungi were compared with the model white rot fungi, *Phanerochaete*

chrysosporium obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India. The cellulolytic enzyme assay was carried out in cellulolysis basal medium (CBM) incorporated with 4% w/v cellulose and 1.6% w/v agar and inoculated with test fungus. The plates were incubated at 25°C and examined for 10 days. Cellulolysis was assessed based on the clearance zones of the opaque agar around growing colonies. Hemicellulolytic enzyme assay by dye staining of xylan agar method was carried out in xylanolysis basal medium by incorporating 4% w/v xylan and 1.6 % w/v agar and inoculated with test fungus. After 3-4 days, the agar plates were flooded with iodine stain. Later, the iodine stain was removed off and washed with water. Results are interpreted as xylan degradation around the colonies that appears as a yellow-opaque area against a blue/reddish purple colour for undegraded xylan. Laccase assay by guaiacol method was done on plates with special medium supplemented with 0.02% guaiacol (Coll *et al*., 1993), inoculated with the test fungus and incubated at 30°C for 7 days. Laccase positive fungi were selected based on the oxidative polymerization of guaiacol to form reddish brown zones in the medium.

Laccase being the major enzyme, further confirmation of laccase was performed by ABTS agar method where lignolytic basal medium (LBM) supplemented with 0.1% w/v ABTS was inoculated with test fungus and examined for 10 days. 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. Peroxidase (LiP and MnP) assay by Azure B agar clearance method was carried out in lignin modifying enzyme basal medium, supplemented with 0.01% w/v Azure B and 1.6 % w/v agar and inoculated with test fungus. The plates were examined for 10 days. Production of LiP and MnP was recorded with the clearance of blue colored medium.

The syringaldazine well test was performed to study the secretion of lignolytic enzymes Lac, LiP and MnP in a single plate with wells on three sides. LBM medium was prepared as earlier, inoculated with the test fungus and plates were left for 5-7 days for growth of cultures. Later, on the fully grown fungal culture in the agar medium, 5 mm wells were formed on three sides. To test for laccase activity, few drops of 0.1 % w/v syringaldazine (in 95% ethanol) were added to first well. To test for peroxidase activity, few drops of 0.1% w/v syringaldazine (in 95% ethanol) plus a few drops of 05 % w/v aqueous H_2O_2 solution was added to the second well. To the third well, 95% ethanol was added (control). The appearance of a purple color around each well within 30 minutes indicates laccase enzyme production. Negative reaction is considered for strong LiP and MnP activity if a positive reaction is observed in Azure B agar test.

2.4.3 Production of laccase, LiP and MnP by selected white rot fungi at different day's interval, temperatures and pH under *in vitro* condition

Based on the results of quantitative and qualitative screening, the white rot fungi, *viz*., *P. pulmonarius, P. sajar-caju, S. commune* were further tested for extracellular secretion of major lignolytic enzymes Lac, LiP and MnP at different days, temperature and pH levels, that will facilitate for production of these enzymes in large scale for industrial applications in future (Thiribhuvanamala *et al*., 2015). The liquid broth was prepared (Sayadi and Ellouz, 1995; Vaidyanathan *et al*., 2011) and the white rot fungi, *P. pulmonarius, P. sajar-caju, S. commune* were inoculated. The secretion of lignolytic enzymes were assessed at different pH (3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) and at different temperatures (20, 25, 30, 35, 40, 45, 50 and 55°C) and different time interval (1st day up to 15th day). The aliquots of culture filtrates were collected and analyzed for the peak activity of lignolytic enzyme production at different pH and temperature as per the method described earlier in liquid screening.

The laccase activity was assayed using 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) used as the substrate (Rasera *et al*., 2009). The assay for the lignin peroxidase activity was estimated using the method of Kang *et al*. (1993) with pyrogallol as substrate. The manganese peroxidase (MnP) activity was assayed as per the method of Wariishi *et al*. (1992) with sodium malonate as substrate as mentioned above.

3. Results and Discussion

3.1 Collection and characterization of white rot fungi

The Western Ghats biosphere region in Indian subcontinent is one of the globally discerned biodiversity hotspots that have an enormous incredible wealth of natural resources. Though the natural diversity of mushrooms is of diverse nature in India; they are not well studied, as the collections of sporocarphic fungi began in India four decades ago (Rahalkar and Sathe, 1976; Sarbhoy, 1997). Nearly 6,500 collections of mushroom flora belonging to 223 species, 59 genera and 15 families of Agaricales have been reported in Northern India by Lakhanpal (1997). These data on the diversity and distribution of the edible and wild mushrooms reveals the richness of mycoprotein in the nation. Not only in terms of edibility, there lie tremendous applications of these mushrooms for bioremediation, biodegradation, biopesticidal and pharmacological values that could be exploited. In this present study, a total of 16 white rot fungi belonged to 6 genera were collected and identified morphologically, were as per the keys provided by the Directorate of Mushroom Research, Solan, India and molecularly characterized as *P. pulmonarius, P. sajor-caju, P. cyctidiosus, P. ostreatus, P. djamor* (C), *P. djamor* (N), *T. versicolor, T. ljubarskii, T. ljubarskii* (C), *Trametes* sp, *P. sanguineus, L. crinitus, L. edodes, L. squarrosulus, S. commune* and *G. gibbosum* (Table 1). It was observed that the climatic conditions prevailing in the study area of Western Ghats favored the occurrence of diverse mushrooms.

3.2 Screening of white rot fungi for lignolytic enzymes

Quantitative assays provide information on the levels of lignocellosic enzyme patterns of fungi, whereas qualitative assays are powerful tools that will provide valuable information to further confirm the particular enzymes secreted. Qualitative assays are further used to screen the enzymes when large numbers of fungi are involved. Certainly, the quantitative assays are used frequently to identify potential organisms. In the present study, through quantitative liquid screening for major lignolytic enzymes, *viz*., laccase, LiP and MnP, it is observed that the fungi isolated were capable of producing substantial quantities of the test enzymes (Figure 1).

Table 1: Morphological and molecular characterization of white rot fungi isolated from Western Ghats of Tamil Nadu

S. No.	Name of the white rot fungi	Place of collection	Source of collection	Morphological characters	Molecular characters (M.W.)	GenBank Accession number
1.	*Pleurotus pulmonarius*	Nilgiris	Dead woods of Eucalyptus tree	Sporophore fleshy with whitish thick stipe with 2.5 cm length, with eccentric attachment, gills decurrent running along the stipe. Pileus whitish to pale tan, 3.5-4.0 cm diameter, smooth, convex to lung shaped within rolled wavy margin.	623 bp	JN 831438.1
2.	*Pleurotus sajor-caju (Syn: Lentinus sajarcaju)*	Nilgiris	Dead woods of Eucalyptus tree	Sporophore with smooth short stipe with 1.5 cm length. Pileus pale white, smooth, 5.0 cm diameter with entire margins. Gills decurrent running down along the stipe.	600 bp	JN 849390.1
3.	*Pleurotus cystidiosus*	Coimbatore	TNAU wood logs	Sporophore with stipe thick, fleshy with 4.0 cm in length. Pileus grey color, fleshy funnel shaped with measured 5.9 cm diameter.	635 bp	JQ 806417.1
4.	*Pleurotus ostreatus*	Nilgiris	Dead woods of Eucalyptus tree	Sporophore with thick short stipe, fleshy and with 3.0 cm in length. Pileus pale whitish to grey color, fleshy funnel shaped with 3.0 cm diameter.	632 bp	JN 807335.1
5.	*Pleurotus djamor* (C)	Coimbatore	Dead woods	Sporophore with thick short stipe with 1.0 cm in length. Pileus pale pink, slightly leathery, lobed margins , 5.0 cm diameter. Gills decurrent running down along the stipe.	637 bp	JX 625130.1
6.	*Pleurotus djamor* (N)	Nilgiris	Dead woods	Sporophore with thick short stipe with 1.0 cm in length. Pileus pale pink, slightly leathery, lobed margins with 4.0 cm diameter. Gills decurrent running down along the stipe.	560 bp	JX 262249.1
7.	*Trametes versicolor*	Siruvani	Dead woods	Sporophores white, continuous with concentric rings mostly deposited by green algae. Pileus with 6.0-9.0 cm in diameter, indented with conspicuous pores on underside, with conspicuous spores on underside. characteristic lumpy excrescences at the point of attachment of stipe to pileus.	540 bp	JN 849391.1
8.	*Trametes ljubarskii*	Coimbatore	Broad leaved dead woods	Sporophores continuous, leathery, light pinkish brown, with concentric rings. Pileus with 5.0-6.0 cm in diameter, indented with conspicuous pores on underside.	520bp	JN831439.1
9.	*Trametes ljubarskii* (C)	Coimbatore	Wood logs	Sporophore single, leathery, 3.0-9.0 g, cream colour, conspicuous rings on pileus, stipe centrally attached, pale whitish in color.	573 bp	JQ 806418.1

10.	*Trametes* sp.	Anaikatti	Dead woods	Light brown sporophores seen spreading flattened on dead woods prominent minute pores.	581 bp	JN 849392.1
11.	*Pycnoporus sanguineus*	Anaikatti	Dead woods	Sporophores in groups, bright orange in color, leathery, pileus is 3.0 -4.0 cm in diameter with very short stipe, laterally attached. Sporophores have concentric zones and with minute pore on the underside.	589 bp	JN 704807.1
12.	*Lentinula crinitus*	Anaikatti	Dead woods	Pileus pale white with 3.0-5.0 cm diameter, humped cap, sinuous, pleated margins; centrally attached stipe white color with 7.0-9.0 cm length.	620 bp	JN 692271.1
13.	*Lentinula edodes*	Nilgiris	Dried bark of silver oak tree.	Pileus smooth light brown in colour, convex 3.5 cm diameter. Stipe brownish white, centrally attached, 3.0 cm in length sub distant gills free observed.	560 bp	JN 790683.1
14.	*Lentinus squarrosulus*	Anaikatti	Dead wood barks.	Pileus pale white concave, entire, scurfy with concentric arrangement of scales. Stipe thick white, centrally attached.	632 bp	JX 625131.1
15.	*Schizophyllum commune*	Kotagiri	Wood logs of silver oak tree	Sporophores in group, tiny, whitish to grey, bracket to fan-shaped without a stipe. Pileus fleshy when young later tough and leathery, under surface composed of split gills.	546 bp	JX 885999.1
16.	*Ganoderma gibbosum*	Anaikatti	Dead woods of broad leaved tree	Sporophores in groups, small narrow, yellowish with white colour at tip. Stipe reddish brown found attached to roots of live broad wood trees and also dead woods.	596 bp	JN 655531.1

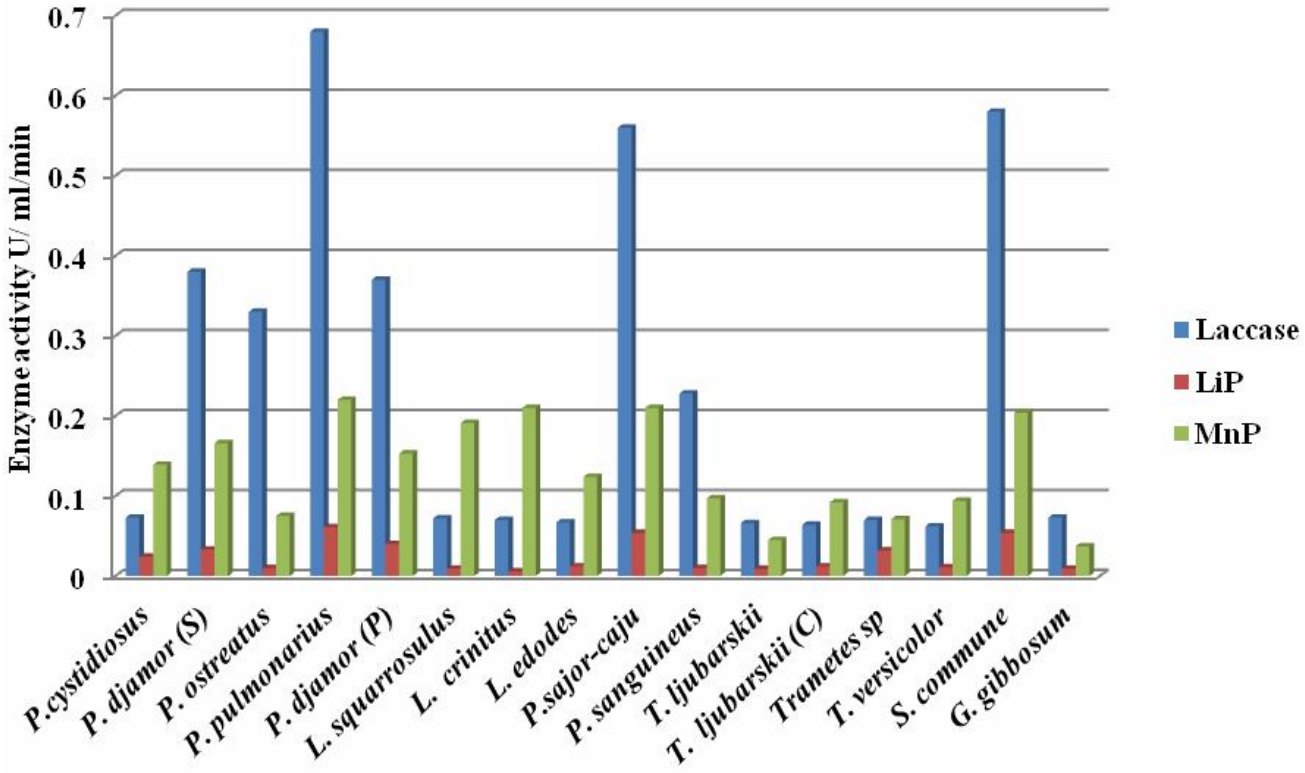


Figure 1: Screening of white rot fungi for major extracellular lignolytic enzyme production

The qualitative screening test revealed that among the 16 white rot fungi tested, *P. pulmonarius*, *P. sajar caju* and *S. commune* secreted significant levels of laccase (0.68, 0.56 and 0.58 U ml^{-1} min^{-1}, respectively), lignin peroxidase (0.061, 0.054 and 0.054 U ml^{-1} min^{-1}, respectively) and manganese peroxidase (0.22, 0.21 and 0.204 U ml^{-1} min^{-1} respectively). The results further depict that apart from Lac, MnP is the second major enzyme secreted, followed by LiP. Also, *P. djamor (S)*, *P. djamor (P)* and *P. ostreatus* were also found to secrete optimum levels of laccase.

Dyes containing lignin properties are normally used in qualitative assays as substrates to screen various fungi for lignolytic enzymes. It is suggested that the adsorption and binding of dyes to the fungal hyphae and further enzymatic degradation by extracellular enzymes serves as major mechanisms for the decolourization activity (Knapp *et al.*,1995; Selvam *et al.*, 2003). Previous studies include screening with various kinds of azo, anthraquinone, heterocyclic, triphenyl methane and polymeric dyes to detect the enzyme secretion by *P. chrysosporium* (Paszezynski *et al.*, 1991). Though laccase can oxidise a variety of substrates like phenolic compounds and aromatic amines, indicators like guaiacol are widely used as substrates for laccase production (Coll *et al.*, 1993; Vaidyanathan *et al.*, 2011). Also inducers like ABTS have been widely used which forms green color in the growth medium due to the oxidation of ABTS 2,22 - Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) to ABTS-azine in the presence of laccase. In our study, secretion of cellulase enzyme was observed by the clearance of cellulose substrate as opaque areas which was prominently visible in *P. pulmonarius*, *P. sajar-caju*, *P. sanguineus*, *P. ostreatus*, *G. gibbosum* and *S. commune* (Figure 2a). High cellulolytic activity of *P. ostreatus* was observed, compared to *P. florida* as reported by Garcha *et al.* (1991). Similarly, xylan degradation around the colonies appeared as yellow opaque area against a blue / reddish purple color which was highly visible in *T. ijubarskii*, *L. crinitus*, *P. sanguineus* and *G. gibbosum* against a blue back ground which revealed the hemicellulolytic activity (Figure 2b). Positive reaction indicated the presence of endoxylanase and β xylosidase enzymes. Laccase assay with guaiacol showed the oxidative polymerization of guaiacol to form reddish brown zone which revealed the production of laccase. Positive results for laccase were prominent in *P. pulmonarius* and *P. sajor-caju* in guaiacol amended medium as observed by production of reddish brown zone whereas in ABTS amended medium, *P. pulmonarius*, *P. sajor-caju* and *S. commune* developed intense green color (Figure 2c). In Azure B agar clearance test, decolorization of Azure B dye was prominent, *i.e.*, complete decolorization by *P. pulmonarius*, *P. sajar-caju* and *S. commune* and *T. ijubarskii* which is positively correlated with the production of lignin peroxidase and manganese peroxidase (Figure 2d). However, ABTS agar method could be interpreted with the development of green color for the positive laccase producing fungi only if a negative reaction or weak reaction is obtained in Azure B agar clearance methods as it is specific for the detection of peroxidase type of lignin modifying enzymes. So, in our study also, the qualitative assay further confirmed that laccase is the major enzyme produced, followed by peroxidase type of enzymes LiP and MnP.

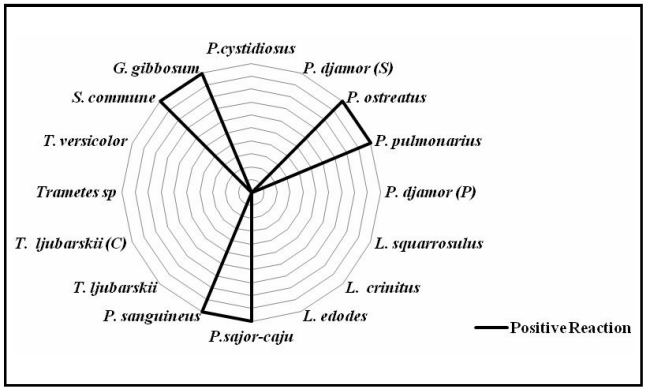


Figure 2(a): Cellulose agar clearance assay

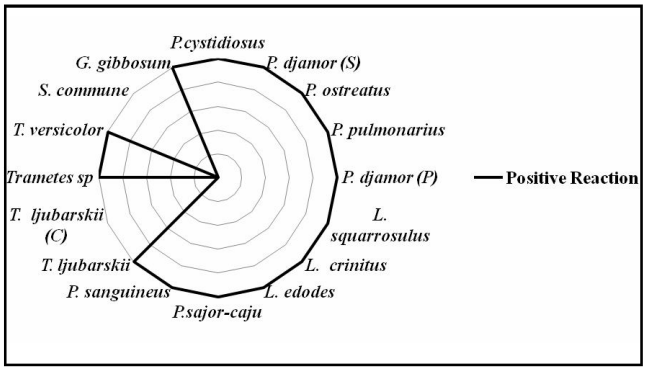


Figure 2(b): Hemicellulose enzyme assay by dye staining of xylan agar method

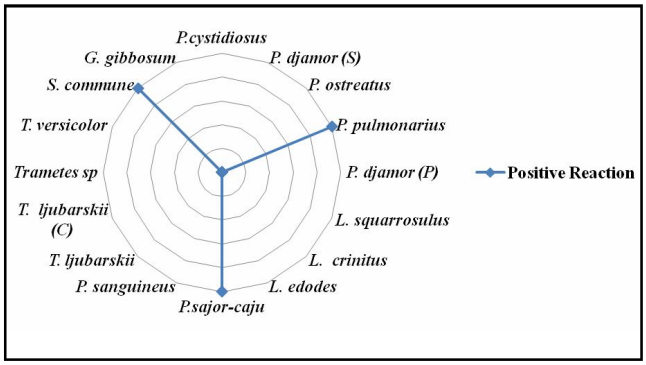


Figure 2(c): Laccase enzyme activity by guaiacol and ABTS method

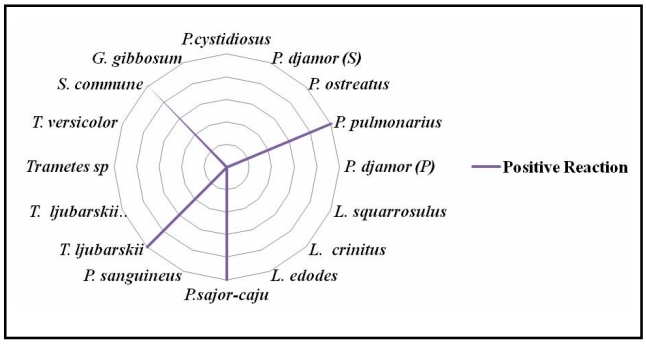


Figure 2d: Azure B agar clearance method for LiP and MnP enzymes

The Syringaldazine well test indicated the secretion of laccase by *P. pulmonarius* and *P. sajor-caju* as observed by the appearance of purple color around each well (Figure 2e) whereas the faint pink colour in *S. commune* suggests enhanced levels of LiP and MnP than laccase. Similarly, Devi *et al*. (2012) reported that the fungus *P. ostreatus* possesses a significant dye degradation capacity and further can be applied in bioremediation of toxic industrial dyes. The results from ABTS agar method could be interpreted with the development of green color for the positive laccase producing fungi only if a negative reaction or weak reaction is obtained in Azure B agar clearance method. Based on the above results, owing to their strong oxidative enzyme production, *P. pulmonarius*, *P. sajar-caju* and *S. commune* were selected to study the secretion of Lac, LiP and MnP enzymes under different pH and temperature over a period of time. Though qualitative assays give an idea about the type of enzyme secreted, quantitative assays always gives a precise description of the enzymes. Seshikala and Singara Charya (2012) reported that among 37 white rot fungi screened, *Stereum ostrea* produced laccase, MnP, LiP, *Tremella frondosa* produced laccase, MnP, LiP and *Trametes versicolor* secreted both MnP and LiP. Jong *et al*. (1992) also reported that 18 fungi out of 20 screened produced MnP and laccase but their decolourization rate varied based on the substrate used. Like in our study, Swamy and Ramsay (1999) reported that in the white rot fungus, *T. versicolor* produced laccase and manganese peroxidase (MnP) that could decolorize the amaranth azo dye. In addition, Harisha *et al*. (2012) identified *Peniophora* sp. hpF-04 and *Phellinus* sp. hp F-17 as the predominant laccase producing strain as they could decolourize more than 80% of decolorization of six to nine types of textile dyes. Similar results were also observed by Erden *et al*. (2009) under solid screening with *G. carnosum* and *T. versicolor* where they found that the decolorization of remazol marine blue effectively while *P. ostreatus* could not decolorize the dye, whereas in *Abortiporus biennis*, there was a transformation of dye.

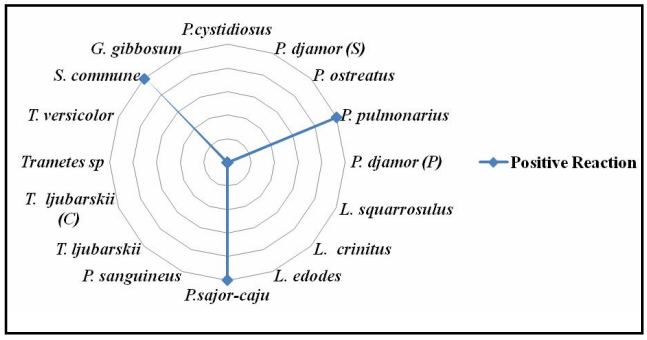
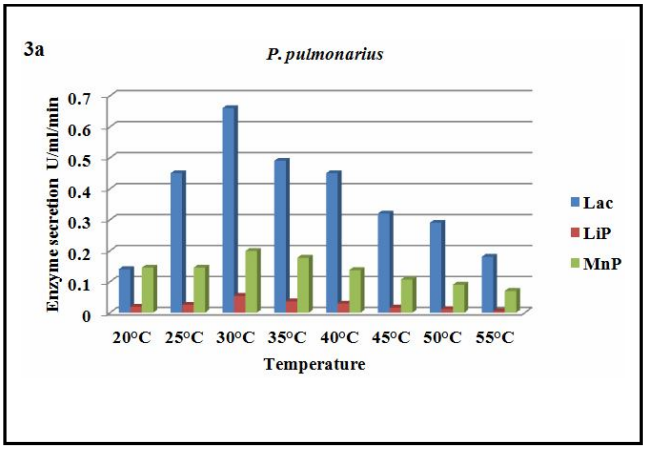


Figure 2(e): Syringaldazine well test for lignolytic enzymes

In nature, the colonization of the white rot fungi is dependent not only on the nature of substrate, but however the secretion of lignolytic enzymes is dependent on pH of the substrate and prevailing temperature conditions. In this aspect, it will be useful to study the extracellular secretion of the major enzymes, *viz*., Lac, LiP and MnP under optimum pH and temperature over a period of time so as to tap higher levels of these enzymes for industrial uses. Perusal of literature also shows that majority of white rots grow optimally at acidic pH values in the range of 4-4.5 (Knapp *et al*., 2001). Temperature also plays an important role in the growth of mycelium and enzyme production, which further influences the enzymatic decolourization rate of the substrate. Though, there is fairly increased enzyme secretion at higher temperatures for dye decolorization, degradation of enzymes also should be taken in to account as it varies from fungi to fungi. Hence, optimum temperature range of 27-30°C, is considered suitable for enzyme production as observed by Erden *et al*. (2009) where he reported that *P. ostreatus* produced Lac and MnP at 26°C on 10th day of incubation whereas *G. carnosum* produced more of MnP and laccase at 4th and 10th day. Similarly, in our study, among the different pH levels tested, laccase activity reached maximum at pH 4.5 and LiP and MnP activity was maximum at pH 5.0 for all the three fungi. The laccase and LiP activity is maximum at pH of 4.5 and MnP activity maximum at pH of 5.0 by *P. pulmonarius*, *P. sajar-caju* and *S. commune* (Figures 3a, b and c). The effect of temperature showed that the laccase, LiP and MnP activity was maximum at 30°C for *P. pulmonarius*, *P. sajor-caju* and *S. commune* (Figures 4a, b and c). Laccase attained their peak enzyme activity on 4th day after inoculation while LiP and MnP attained maximum activity on 7th and 8th day for *P. pulmonarius* and *P. sajor-caju*, respectively. In case of *S. commune*, laccase attained maximum on 10th to 12th DAI while LiP and MnP on 6th and 7th DAI, respectively (Figures 5a, b and c). The results showed that *P. pulmonarius* produced maximum lignolytic enzyme production at pH of 4.5 and temperature of 30-35°C. Moreover, the substrates also play a major role in induction of exracellular enzymes as noticed in case of *P. pulmonarius*, wherein the fungus produced significant levels of laccase and LiP in different farm agrowastes, *viz*., sugarcane, banana, millets, pulses, cocopeat, cocoa wastes coir pith and oil palm bunch wastes from 21st to 28th day after inoculation under solid fermentation (Thiribhuvanamala *et al*., 2013). Extracellular enzymes like amylase, cellulase, glucose isomerase, laccase, lipase, protease and xylanase, *etc*., are used in different segments such as agrowaste degradation, coir bleaching, distilleries, detergents, food, feed, fuel, pharmaceutical and dye industries (Li *et al*., 2012). Recently, white rot fungi have exploited much in several industries due to its ability in bioremediation processes like biobleaching, biocuring, and biopulping (Iqbal *et al*., 2013). Lignocellulolytic enzymes extracted from these organisms become a believable source for cellulosic biodistileries, biorefineries and bioethanol production.



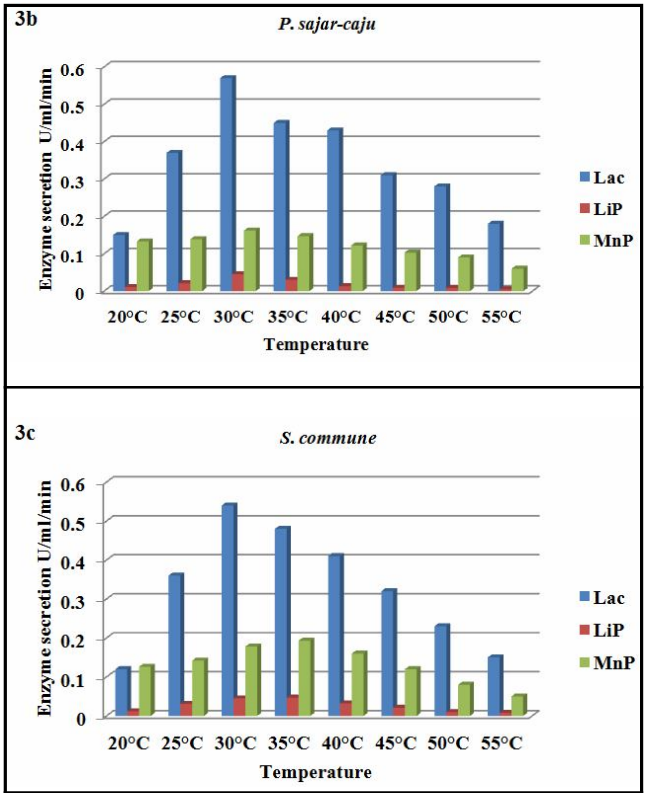
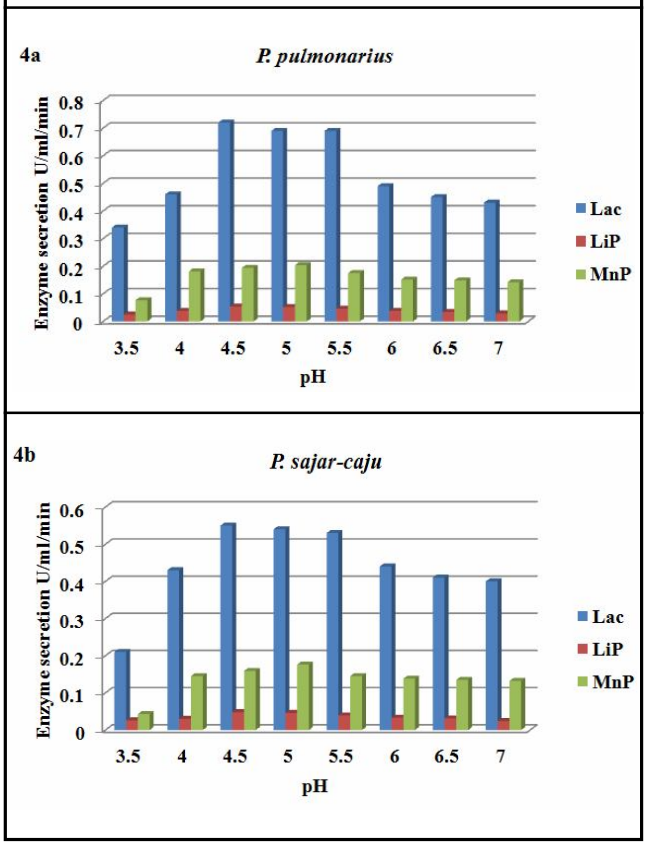


Figure 3: Production of laccase, LiP and MnP by selected white rot fungi at different temperature



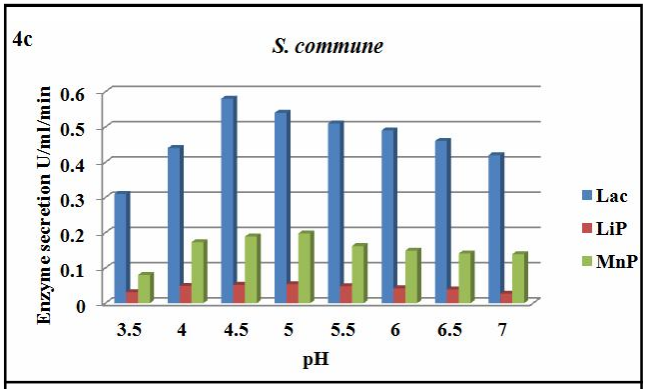


Figure 4: Production of laccase, LiP and MnP by selected white rot fungi at different pH

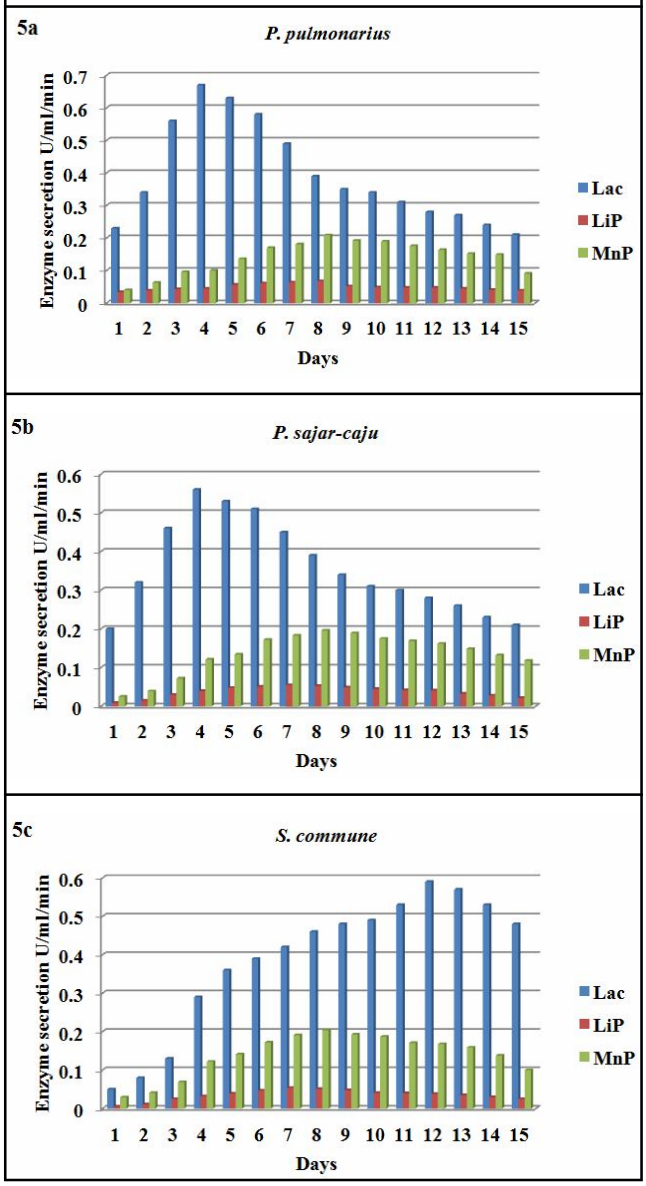


Figure 5: Production of laccase, LiP and MnP by selected white rot fungi at different day's interval

4. Conclusion

The results obtained in this study indicated that the lignocellulolytic enzyme secretion pattern by diverse white rot fungi belonging to six genera; *Pleurotus* spp, *Lentinus* spp, *Ganoderma* spp, *Trametes* spp, *Pycnoporus* spp and *Schizophyllum* spp. at different temperatures and pH over a period of time. The *P. pulmonarius* and *P. sajar caju* being edible and utilized for commercial production can also find application in biodegradation of agrowaste by the farming community that will enhance the soil health. Certainly this data will be quite useful to utilize these enzymes in future for various industrial applications especially to produce large quantities of these enzymes for bioremediation of industrial effluents and biodegradation of agrowastes.

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Conflict of interest

We declare that we have no conflict of interest.

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