

# *An Exploration of Jaipur's Fungal Biodiversity for Ligninolytic Potential*

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**Abstract:** With the growing emphasis on sustainable development, there is an urgent need to be more concerned about the conservation, management and exploitation of microbial diversity so as to develop ecofriendly technologies for various industries. From this viewpoint, fungus has been the relatively less explored group although the fungi possess a huge number of enzymatic activities which can be easily used to replace different chemicals in various industries for lowering down their pollution levels. Fungi have diverse capacities to biotransform and to completely destroy toxic chemicals viz. pesticides/xenobiotics etc. from our environment. Their ligninolytic enzyme complex is reported to be the best for degradation of such pollutants. This enzyme system is also reported to be involved in the degradation of highly recalcitrant lignin biomolecule. Basidiomycete group of fungi especially white rot fungi are reported to be the best microorganisms for lignin degradation due to their unique ability to degrade and mineralize lignin through secretion of extracellular ligninolytic enzymes, having high tolerance to toxic substances and high degree of non-specificity in their nature. The potent cultures of white rot fungi having high ligninolytic potential can be very useful for various applications like biopulping/biobleaching/effluent treatment etc. for the paper industry.

Keeping in view of the above, present study was taken up to isolate various white rot fungal cultures from different regions of the pink city. Further, the effective qualitative screening of the obtained isolates was carried out by growing them in the PDA plates supplemented with the standard chromogenic substrates (ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)], Tannic Acid, Guaiacol and RBBR (Remazol Brilliant Blue)) followed by their microscopic examination. Thus, we were able to isolate thirteen number of white rot fungal cultures having ability to produce ligninolytic enzymes. Out of these thirteen cultures, seven have shown positive result to ABTS and Guaiacol, nine cultures showed positive response for the tannic acid while the four cultures were shown to be positive to RBBR screening test. There were only two isolates which were positive to all the four screening tests whereas four isolates were found to be negative to all of them. Thus from the present screening of the white rot fungal isolates of Jaipur city, it was found that nine isolates have ligninolytic capability. Further studies on the quantitative estimation of their

enzymes are being carried out in terms of the enzyme profiling of all the isolates.

**Keywords:** Lignin, White rot fungi, Lignin peroxidases, Manganese peroxidases, Laccases, Guaiacol, RBBR, ABTS, Tannic Acid

## I. INTRODUCTION

From the beginning of life on earth, microbial diversity has been very important as microbes perform numerous essential functions of the biosphere including nutrient recycling and environmental detoxification. Moreover, they also have massive commercial and industrial applications. Therefore, a proper management and exploitation of the microbial diversity can play a very important role in sustainable development. However, in view of their contributions in national economy, health and quality life improvement a very little efforts have been made.

Fungal diversity and their natural beauty occupy prime place in the biological world. India has been the fountainhead for such fungi. Only a fraction of total fungal wealth has been subjected to scientific analysis and mycologists have to untangle the unexplored and hidden wealth. Around one third of fungal diversity of the world exists in India. About 50% of 1.5 million of fungi are characterized until now and unfortunately about only 5 -10% of fungi can be cultured artificially (Manoharachary et al. 2005). On the basis of cellulolytic and ligninolytic activity the fungal species as well as strains vary considerably as these organisms are distributed widely in forests throughout the world. White rot fungi are the most common fungi which degrade wood as their degradative process have been of great interest for their ability to degrade all cell wall including lignin (Akhtar et al. 1997; Kirk and Cullen, 1998; Martinez et al. 2005; Dashtban et al. 2010). Lignin is the second most abundant deposit organic carbon in the biosphere, being surpassed only by cellulose. It is a highly irregular polymer on earth with an undefined structure. It is composed of

oxygenated phenyl-propanoid units which are linked among them through various types of C-C and C-O-C bonds. Because of these unusual features this macromolecule is very difficult to be degraded. White rot fungi is the only group of microorganisms which have the capacity to breakdown lignin extensively to carbon dioxide and water. This fungal group has a capacity to degrade lignin selectively through the secretion of a set of extracellular enzymes which are directly involved in lignin degradation. The enzymes secreted are a phenol oxidase termed Laccase, Lignin peroxidase (LiP) and Manganese peroxidase (MnP) (Lobos et al. 2001). This ability of selective lignin degradation make them useful for various biotechnological uses, such as bioremediation, biobleaching of pulp, biopulping and pretreatment of biomass for bioenergy production (Blanchette et al. 1988; Lobos et al, 2001). The goal of present study is to isolate potent ligninolytic isolates of white rot fungi from different areas of the pink city, Jaipur for the development of some possible biotechnological applications in pulp and paper industry particularly the handmade paper industry.

## II. MATERIALS AND METHODS

Potato Dextrose Agar (PDA) was used to isolate fungi from the samples. 0.01% of chloramphenicol was added to the autoclaved media aseptically to inhibit the growth of bacteria. In addition to this benomyl, a benzimidazole fungicide, was added prior to autoclaving in order to select the wood decaying fungi (Maloy, 1974). Lactophenol cotton blue was used to stain fungal culture for microscopy and cork borer as an inoculating tool for culture inoculation. Beside this, chromogenic substrates were also used for their qualitative ligninolytic screening. The standard cultures were procured from IMTECH Chandigarh for comparative study of ligninolytic activity of isolates with them.

### A. Sample Collection

Different samples were collected in sterile culture bottles as well as in zip lock poly bags from different natural habitats. Sample collection was done during the rainy season as well as in summers too. The samples were collected in three forms that is solid, liquid and semi solid. The solid samples including scraped tree bark, fruiting bodies, decayed pulp and soil sample while the liquid and semi liquid waste included pulp waste with water and waste water. The sampling sites included Community parks, Kulish Smiriti Van (Jaipur), Cowshed (Sanganer Goshala, Jaipur), Handmade Paper Decayed Wood sample (KHPI), Paper Industry Pulp Waste, Decayed Pulp and Sanganer domestic dump waste. Thus, an effort

has been made to explore myco-biodiversity of Jaipur for their ligninolytic potential.

### B. Media Preparation and Fungal Purification

#### 1) Media Preparation and Sample Fungal Inoculation

Potato Dextrose media with different concentrations of benomyl viz. 1%, 0.75%, 0.50%, 0.25% and 0.1% was used for fungal inoculation with constant concentration of chloramphenicol (0.01% (w/v) (Maloy, 1974; Kiiskinen, 2004). Inoculation was done by using two methods viz. direct and dilution techniques. A solid sample that is fruiting body was inoculated using direct method while the soil and liquid samples were used to inoculate through dilution method using spreading technique. After inoculation, the plates were incubated in an incubator at 36°-38°C for 5-7 days or till the growth was seen.

#### 2) Isolation and Purification of Fungus

The isolated cultures were sub-cultured further through spore serial dilution method with agar plate spreading technique till the pure colonies were obtained. As the pure colonies of same morphology were obtained, they were used to inoculate on the centre of the plain PDA (without benomyl) plate using cork borer as an inoculating tool (Fusaro, 1972).

### C. Colony Morphology and Microscopy of Isolated Cultures

Different fungal cultures produce different types of colonies. Some colonies may be circular or irregular in shape while some are filliform. Therefore, colony morphological study was done for all the pure isolated fungal cultures in terms of temperature, forms, elevation, margin, surface, color and hyphae (Watanabe, 2002). The isolated cultures were studied microscopically for their identification under the light microscope using lactophenol cotton blue stain. Presence of septate hyphae and clamp connection, the identification features of white rot fungi were tried to find out among all the isolated cultures so that it could be judged whether they belong to white rot fungi or not (Watanabe, 2002).

### D. Cultivation on Solid Media

The isolated pure cultures were used to cultivate on plain PDA plates and the fully-grown plates were preserved at 4°C for further transfer into slants and plates.

*E. Qualitative Screening of Fungal Isolates for Ligninolytic Enzymes*

Screening was performed using the freshly grown fungal isolates on plain PDA media. For screening purpose, PDA was substituted with ligninolytic enzyme substrate or a ligninolytic indicator compound viz. 0.5% tannic acid (TA), 0.01% guaiacol (GU), 2mM (2,2-Azinobis-3-ethylbenzthiazoline-6-sulphonate (ABTS) and 0.04% Remazol brilliant Blue R (RBBR) (Gnanasalomi and Gnanadoss, 2013; Pointing, 1999; Kiiskinen et al.,2004).

III. RESULTS AND OBSERVATION

Naturally occurring microorganisms having the ability to produce various enzymes. At present, most of the enzymes are important for human welfare and having different industrial applications. In the present study, the fungal culture was isolated from various sampling sites which are either rich in lignin or having lignin as a waste, lignin was degraded by the native microbes that are growing over the waste. In such a way, it is fact that the microbes which are isolated from sampling sites may have ability to produce ligninolytic enzymes.

A. Isolation of fungal culture

The incubated PDA plates were observed daily for fungal growth. Thirteen new fungal cultures have been isolated from the source samples via continue culture-observation and the standard sub culturing techniques.

B. Morphological and microscopic study of colony

The morphological study of fungal cultures was done on the basis of their growth pattern in petri plates in accordance of the parameters (Table-1)as described earlier. The microscopic study was also carried out for the isolates which resulted that among the 13 isolates, 9 isolates showed the presence of clamp connection, the key feature of basidiomycetes, thereby indicating that they belong to basidiomycetes sub-division (Figure. 1).

C. Screening of fungal culture

For the screening of their ligninolytic enzyme production abilities, suitable tests were performed using ABTS, Guaiacol, Tannic acid and Remazole Brilliant Blue (RBBR) as the chromogenic substrates. Colonies showing green, dark green, purple zone or colored media below colonies in the presence of ABTS, reddish brown colour in guaiacol, dark brown

halo in tannic acid and appearance of clear zones around colonies in RBBR indicated the presence of ligninolytic enzymes and the lignin degrading activity of the isolate used. (Figure. 2-11).

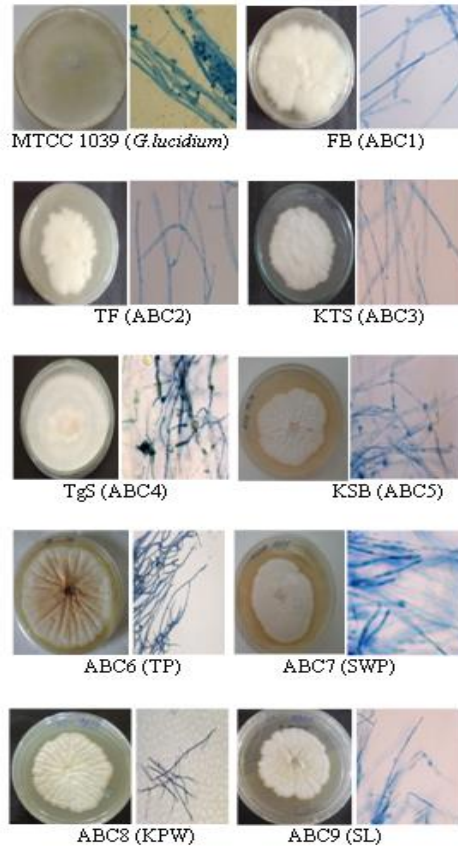


Fig. 1: Microscopic View of Standard Culture (MTCC 1039) and Isolated Fungal Cultures

Table 1: Morphological Characteristics of Isolated Fungal Cultures.

S.No.	Fungal isolate	Temperature and incubation period	Form	Elevation	Margin	Surface	Color	Hypha (Microscopic View)
1	FB (ABC1)	35-40°C 4-5 days	Circular	Convex	Filiform	Convex	White	Septate with clamp connection
2	TF (ABC2)	35-40°C 4-5 days	Irregular	Convex	Filiform	Smooth	White	Septate with clamp connection
3	KTS (ABC3)	35-40°C 4-5 days	Irregular	Raised	Filiform	Smooth	White	Septate with clamp connection
4	TGS (ABC4)	35-40°C 5-6 days	Circular	Flat	Lobate	Dry	White and media converted to pink in color	Septate with clamp connection
5	KSB (ABC5)	35-40°C 5-6 days	Irregular	Flat	Curled	Wrinkled	White	Septate with clamp connection
6	TP (ABC6)	35-40°C 5-7 days	Irregular	Flat	Curled	Wrinkled	White	Septate having clamp connection and basidium
7	SWP (ABC7)	38-40°C 7-8 days	Circular	Convex	Entire	Glistening	White	Septate with clamp connection
8	KPW (ABC8)	37-40°C 5-7 Days	Irregular	Raised,	Filiform	Smooth	White	Septate with clamp connection
9	SL (ABC9)	37-40°C 3-5 Days	Irregular	Flat	Curled	Wrinkled	White	Septate with clamp connection

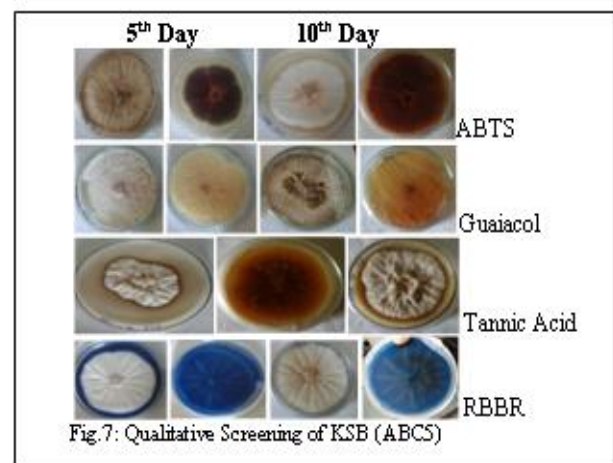
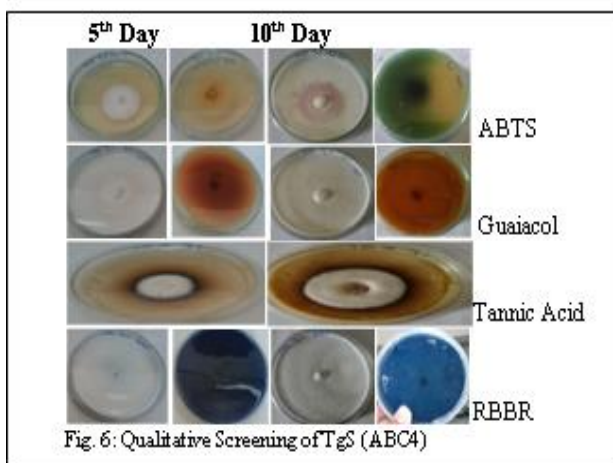
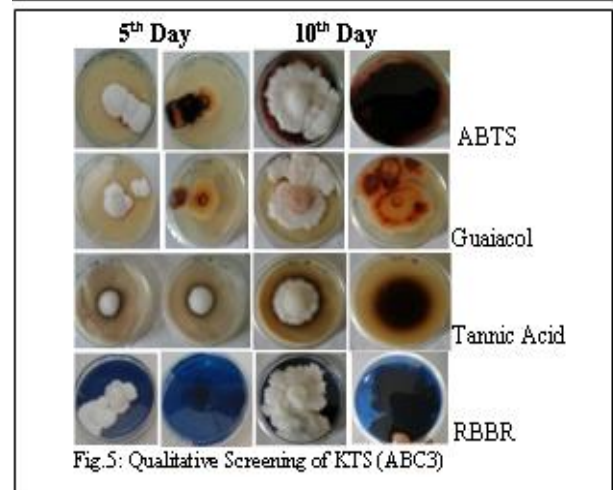
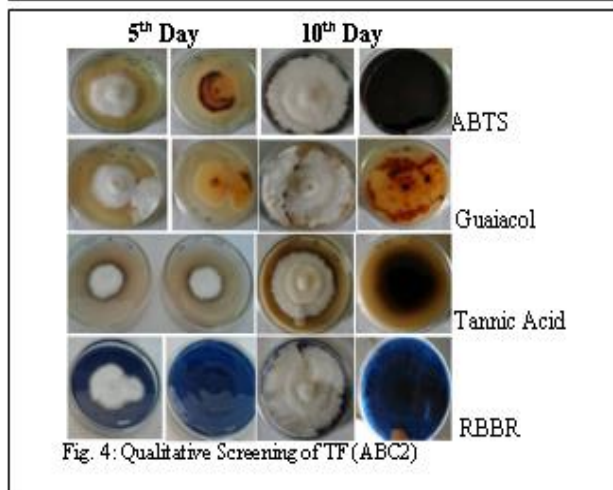
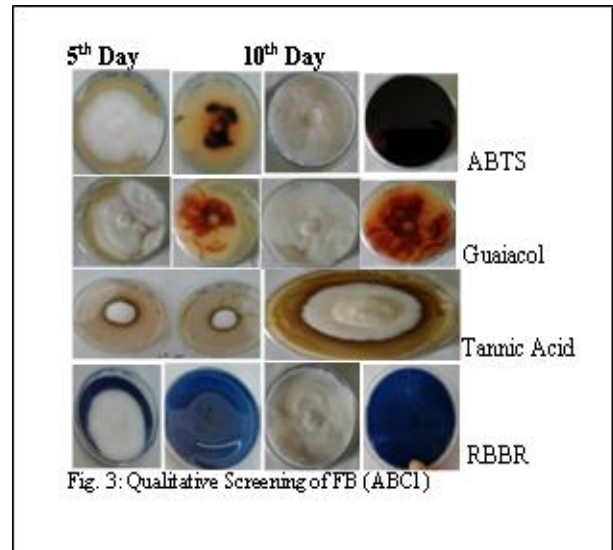
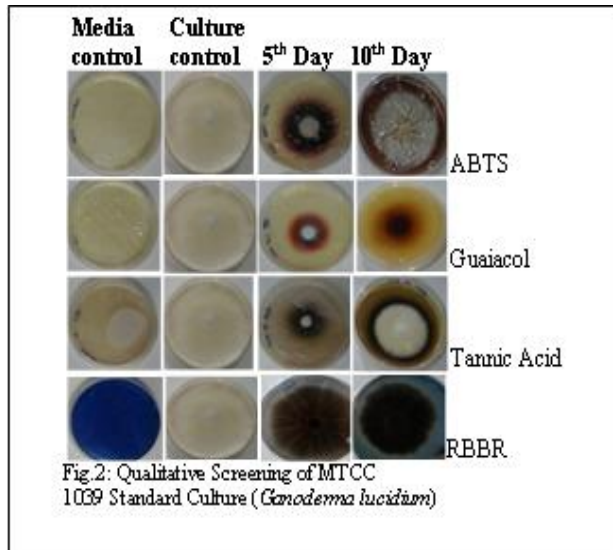


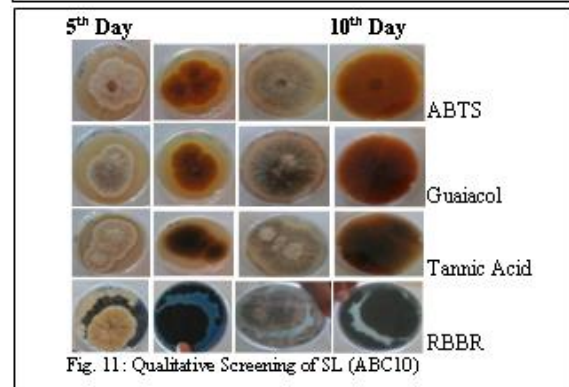
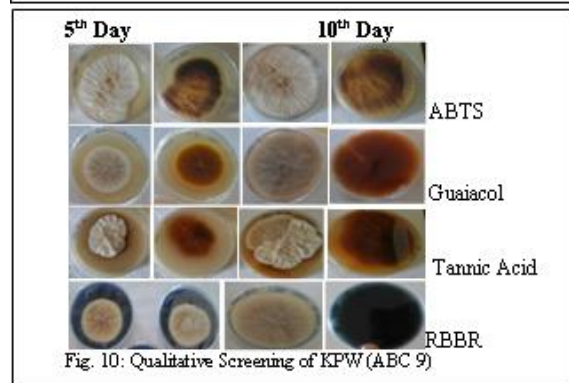
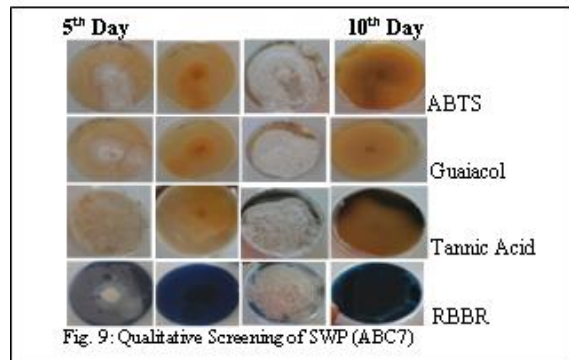
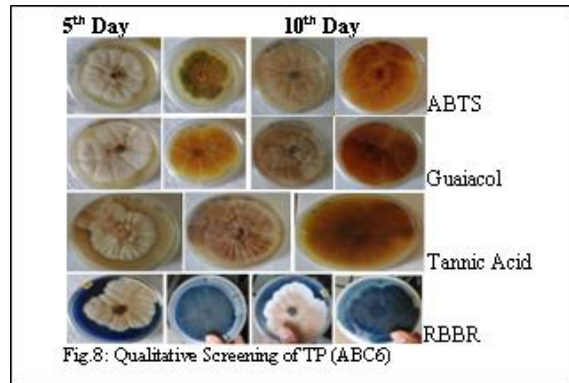
Table 2: Numbers of cultures showing positive and negative results in each screening test

Screening test	Number cultures shows positive and negative result	
	Positive	Negative
ABTS	7 (TF, T <sub>g</sub> S, KTS, KSB, FB, TP, KPW)	6 (PSS, MPF, SSI, SWP, SL, KHPI)
Guaiacol	7 (TF, T <sub>g</sub> S, KTS, KSB, FB, TP, KPW)	6 (KSB, PSS, MPF, SSI, SWP, KHPI)
Tannic acid	9 (TF, T <sub>g</sub> S, KTS, FB, TP, KPW, SL)	5 (PSS, MPF, SSI, SWP, KHPI)
RBBR	4 (KSB, TP, KPW, SL)	9 (TF, T <sub>g</sub> S, KTS, FB, PSS, MPF, SSI, SWP, KHPI)
Positive for all test	2 (TP, KPW)	
Negative for all test	5 (PSS, MPF, SSI, SWP, KHPI)	

From the present study, it was concluded that the numbers of cultures showing positive and negative results were varying in each screening test (Table-2). It was found that some of the samples showed better results while the some didn't. The isolates showing better colored zone and better color formation or better result of screening test were considered for further study as the intensity of colored zone production reflects about the level of enzyme production.

#### IV. SIGNIFICANCE OF THE STUDY

The microorganisms commonly occurring in soil, dead and decayed trees, pulp and paper waste etc. have their important role in the carbon circulation and that may be to degrade residual lignin. Such microorganisms seem to use enzymes for lignin degradation. The lignin-degrading microbes and their enzymes can contribute to more efficient and environmentally sound use of renewable lignocellulosic feedstocks for sustainable production of materials, chemicals, biofuels and energy in the industrial applications including paper pulp bleaching in chlorine-free sequences, effluent treatment and biopulping. White rot fungi are considered as the potentially useful agents for biopulping as they contribute to reduce energy and chemicals in mechanical and chemical pulping, thus minimizing the environmental impact of traditional pulping process. A combination of solid state fermentation technology with the capability of WRF to selectively degrade lignin can be very useful for making the applications of lignocelluloses based biotechnologies, possible. Thus, white rot fungi and/or their ligninolytic enzymes can replace the conventional chemical process of several industries including the paper industry.



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