



## **Lignin-Degrading Microorganisms from Organic Soils**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. Author YN designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors M and SBG managed the analyses of the study. Author ST managed the literature searches. All authors read and approved the final manuscript.*

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### **ABSTRACT**

The most prevalent aromatic polymer in nature is lignin, produced by higher plants and thought to make up 30-35 percent of the non-fossil organic carbon on the planet. Lignin hydrolyzing enzymes such as lignin peroxidase, laccase, manganese peroxidase, and others produce a variety of aromatic monomers, including ferulic and vanillic acids. However, very little research has been done on the role of microbes in lignin degradation. In the present work, we have isolated 25 ligninolytic bacteria and 25 ligninolytic fungi from organic soils of Koppal, Raichur districts of Karnataka. The bacterial isolates were identified as *Pseudomonas putida*, *Bacillus subtilis*, based on biochemical tests, and fungi were identified as *Aspergillus niger*, *Trichoderma viridae*, *Phanerochaete chrysosporium* and *Pleurotus ostreatus* based on morphological characters. The ligninolytic activity of bacterial isolates was high when compared to fungal isolates. All the isolates produced detectable amounts of lignin peroxidase, manganese peroxidase, and laccase under *in vitro* conditions. In dye decolorization test, fungal isolates KGST-1, KGST-2, and KKSP could decolorize Ramazol Brilliant Blue R and Congo red.

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## 1. INTRODUCTION

Lignin has been the most fundamentally complex carbohydrate containing a high molecular weight and the most recalcitrant, consisting of multiple biologically stable linkages [1]. It is composed of three main components: cellulose (35-50 percent), hemicelluloses (20-35 percent) and lignin (10-25 percent) [2]. Lignin is the second most common organic biopolymer in nature, after cellulose. It is the biosphere's most concentrated aromatic polymer (Rahman et al. 2013). Lignin is made of phenyl propanoid monomer units [3,3a]. Lignin causes significant pollution and contamination in aquatic environments due to its low biodegradability. Environmental contamination is caused by large volumes of lignocellulosic waste created by forestry and agricultural activities, paper-pulp factories, timber industries, and many other agro-industries [4]. It is a major source of renewable organic matter and a primary renewable natural resource for the planet. Farm biomass is considered waste, but it can be processed into useful items such as biofuels, pesticides, low-cost fermentation sources, enhanced animal feeds, and human nutrients. The structure and function of microbial communities influences the lignocellulose decomposition [5]. Forest soils are the main source of lignocellulose decomposing microorganisms [6,7], however, cultivated lands are dominated by bacteria [8]. Extracellular, oxidative, and unspecific enzymes that can release potentially unstable compounds and then undergo a variety of oxidative reactions catalyze the reaction. The oxidation of lignin has been referred to as "enzymatic oxidation." [9].

Many different types of microorganisms will degrade lignin-rich biomass in nature. Filamentous fungi, such as white rot fungi and other fungi, have the ability to degrade lignin viz., *Collybi*, *Phanerochaete chrysosporium*, *Arthrotrichum*, *Streptomyces viridosporus*, *Lepiota*, *Pleurotus eryngii*, *Poria*, *Trametes trogii*, *Fusarium proliferatum*, *Agaricus*, *Ervernia*, *Copricus*, *Mycema* and *Swtarium* [10]. The bacterial species are little known to degrade lignin [3,3a]. Few bacteria were reported to produce laccase, including *Acinetobacter* [11], *Arthrobacter* [12], *Bacillus* [13], *Branhamella*, *Brochothrix* [13,14], *Micrococcus* [14], *Nocardia* [15] etc., are able to produce polyphenol peroxidases, which can mineralize lignin and lignin-containing compounds (Kawakami, 1975).

The emerging evidence for bacterial lignin degradation highlights the need to classify both bacterial and fungal species and contrast their functions in different soil environments in order to better understand *in situ* the mechanisms that control lignocellulose decomposition [16]. Lignin degrading enzymes are essentially extracellular in nature due to the large and complex structure of lignin, which cannot enter the cell for intracellular action. The lignin degrading abilities of fungi and bacterial isolates was compared on different indicator media. There is a pressing need for the development of rapid biodegradation of lignin with biological agents. In view of this, we have identified some potential areas to work in this category. We aimed to isolate the lignin-degrading bacteria and fungi and characterize them using different media.

## 2. MATERIALS AND METHODS

### 2.1 Isolation of Lignolytic Microorganisms

For the isolation of ligninolytic bacteria and fungi, seventy rhizosphere soil samples were obtained from various crops under organic farming in the Hyderabad Karnataka Region (Raichur, Koppal, and Kalaburagi). On lignin-enriched Nutrient Agar medium (N.A. medium) and Potato Dextrose Agar medium, the ligninolytic bacteria and fungi were isolated (PDA medium). The plates were inoculated and incubated at 28°C for 3-7 days. Eximious microbes that could create a halo zone around the colony were purified and preserved under 4°C until further use. According to Cappuccino and shorman [17] protocols, the isolates were screened for biochemical characters such as catalase, starch hydrolysis, oxidase, MR-VP, H<sub>2</sub>S production denitrification, acid-gas production test, and citrate utilization test.

### 2.2 Screening on Indicator Media

As recommended by Karthikeyan and Sivakumar [18], three indicator media were used. The mediums used were: a) Malt extract tannic acid medium, b) Low nitrogen agar medium with methylene blue, and c) Low nitrogen agar medium with phenol red. The microbes were spotted on indicator media like Malt Extract Tannic Acid Agar Medium (META) and low nitrogen agar medium amended with phenol red

(0.02 %) and methylene blue (0.02 %) separately [18] and incubated at 28°C for 3-5 days. The colonies showing halo zones surrounding them on META medium and low nitrogen with methylene blue medium were considered positive for lignin degradation. The colonies showing red color surrounding them on low nitrogen agar medium amended with phenol red were considered as positive. The isolates positive on all these media were purified and maintained on nutrient agar and potato dextrose agar plates and refrigerated till further use.

### 2.3 Dye Discolorization Test

PDA medium containing 0.04 percent w/v Ramazol Brilliant Blue R (RBBR) (R8001, Sigma), 0.04 percent w/v methyl green (M5015, Sigma), or 0.01 percent w/v guaiacol (G5502, Sigma) was used in dye decolorization experiments. Before sterilization, guaiacol was added to PDA, and RBBR and methyl green were added to sterilized filtered solutions. Each fungal strain was inoculated into all media, incubated at 30°C for two weeks, and the growth was monitored [19].

### 2.4 Estimation of Enzymes Activity

The tannic acid procedure was used to estimate enzymes such as lignin peroxidase (E.C. 1.11.1.14), manganese peroxidase (E.C. 1.11.1.13), and laccase (E.C. 1.10.3.2) [20]. The isolates were inoculated into Malt Extract Tannic Acid (META) agar broth and incubated on a rotary shaker for 10 minutes before centrifuging at 10,000 rpm and extracting the supernatant. The enzyme extract was combined with a 1 % tannic acid solution. To stop the reaction, 90 percent ethanol was applied after 10 minutes of incubation at 30 °C. A colorimeter was used to measure the optical density at 310 nm.

## 3. RESULTS AND DISCUSSION

### 3.1 Isolation and Characterization of Lignin-Degrading Microorganisms from Organic Soils

Out of the seventy samples, twenty-five of each efficient bacterial and fungal isolates were selected based on the capacity for lignin degradation. We got more isolates from Gangavathi, the Basavapattana region, followed by Koppal and Raichur district. Among the twenty-five native bacterial isolates, ten were identified as *Bacillus subtilis* nine as

*Pseudomonas putida* and six were grouped as unidentified species tentatively. Whereas the fungal isolates were characterized as nine of them were seven of them were *Phanerochaete chrysosporium*, three of them were *Pleurotus ostreatus*, three of them were *Aspergillus niger* and the rest (three) of them were unidentified species. The results revealed that all *Bacillus subtilis* were positive for catalase test, starch hydrolysis, oxidase test, V.P. test, and citrate utilization in the biochemical characterization. All the *Pseudomonas putida* were positive for oxidase test, denitrification test, and negative for acid and gas production. All the isolates were negative for the H<sub>2</sub>S production test. Tang et al. (2012) reported that most of the lignin degrading bacteria belongs to *Micrococcus sp.*, *Brevibacterium sp.*, *Arthrobacter sp.* The results are in agreement with the findings of Zeng et al. [21], reported that increase in carbohydrate and lignin ratio.

### 3.2 Lignin Degradation on Indicator Media

Eight bacterial (KGSB-1, KGSP, KGSB-2, KKSB-2, BKSB, RMSP-1, RMSP-2, and RMSB) and eleven fungal isolates ( KGST-1, KGST-2, KGSA, KKST, KKSU-1, KKSP, BKST, RLST-1, RLSP-2, RMSP, and KKSU-2) produced halo zones around the colonies on malt extract tannic acid medium, low nitrogen agar medium with methylene blue and low nitrogen agar with phenol red (Table 1). *Pseudomonas putida*, *Bacillus subtilis*, *Phanerochaete chrysosporium*, and *Pleurotus ostreatus* were found to be positive on the META, LNAM+MB, and LNAM+PR mediums. Sasikumar et al. [22] found lignin solubilization by *Pseudomonas sp.* on minimal salt media containing lignin (MSM-L). A clear halo zones around the colony was observed on indicator media. Yang et al. [23] reported that *Burkholderia sp.* and *Pseudomonas sp.* after days of incubation. *Pseudomonas sp.* and *Bacillus sp.* have the limited capacity to use aromatic aldehydes and produce color on indicator media [24] however, solubilizations were increased upon adding copper sulphate in the growth media.

### 3.3 Dye Decolorization Test

On NA and PDA agar plates, the strains were screened for their ability to decolorize various indicator dyes Remazol Brilliant Blue R (RBBR), Methyl Green (M.G.), Methylene Blue (M.B.), and Congo Red (C.R.) of the respective indicator. The results show that fungal isolates KGST-1,

KGST-2, and KKSP could decolorize RBBR and Congo red even better than the reference strains FR1 and FR2. KGSA and RMSP could efficiently decolorize methylene green along with RBBR. Among the bacterial isolates, KGSB-1 and RMSP-2 could decolorize RBBR even better than reference strains (Table 2). The isolates *Pseudomonas sp.*, *Bacillus sp.*, and *Pandorea sp.*, showed limited dye decolorizing capacity of particularly, Azure B, methylene Blue and Toluidene Blue O was reported by Bandounas et al. [24]. However, a clear dye discoloration was reported by Umashankar et al. [25] by six bacterial isolates.

### 3.4 Enzyme Production

Twenty-five bacterial and fungal isolates of each were grown on selective lignin enriched medium and screened for efficient lignin degradation. Based on O.D. value, eight bacterial strains and eleven fungal strains on par with reference strain were selected for further screening studies. The

bacterial isolate KGSB-2 was found to be superior in the production of enzymes (O.D. value of 1.040) over the controls (Fig. 1). The isolates KGSP, KGSB-1, KKSU-2, BKSB, RMSP-1, RMSP-2, and RMSB were on par with reference strains. While rest all others were lower than the reference strains. Among the twenty-five, fungal isolates KGST-1, KGST-2, KGSA, KKST, KKSU-1, KKSP, BKST, RLST-1, RLSP-1, RLSP-2, and KKSU-2 were on par with reference strains while rest all others were lower than the reference strains (Fig. 2). The production of extracellular enzymes by five bacterial isolates under *in vitro* conditions was confirmed by Bugg et al. [3,3a]. *Pseudomonas fluorescens* produced three dyp-type peroxidases to degrade the lignin compounds under *in vitro* conditions [26].

On liquid media, enzymes such as Lignin Peroxidase (LiP), Manganese Peroxidase (MnP), and Laccase were produced using efficient isolates from preliminary screening. Several of

**Table 1. Lignin degradation test on indicator media**

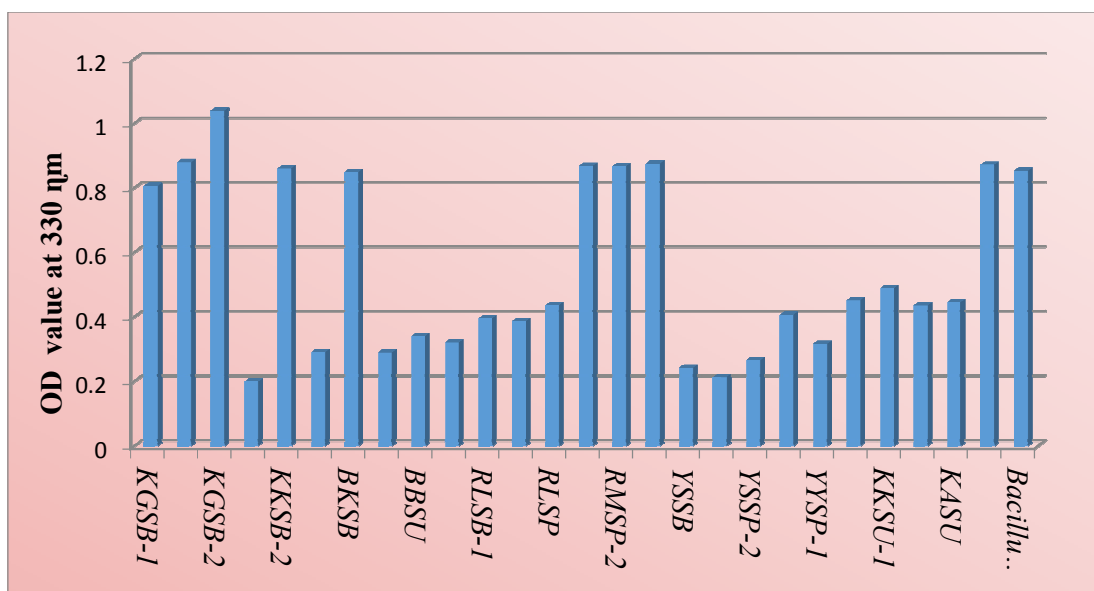
Sl. No.	Isolates	META	LNAM (MB)	LNAM (PR)
<b>a) Fungal Isolates</b>				
1	KGST-1	+	+	+
2	KGST-2	+	+	+
3	KGSA	+	+	+
4	KKST	+	+	+
5	KKSU-1	+	+	+
6	KKSP	+	+	+
7	BKST	+	+	+
8	RLST-1	+	+	+
9	RLSP-2	+	+	+
10	RMSP	+	+	+
11	KKSU-2	+	+	+
12	<i>Phanerochaete chrysosporium</i> *(FR1)	+	+	+
13	<i>Pleurotus ostreatus</i> *(FR2)	+	+	+
<b>b) Bacterial Isolates</b>				
1	KGSB-1	+	+	+
2	KGSP	+	+	+
3	KGSB-2	+	+	+
4	KKSU-2	+	+	+
5	BKSB	+	+	+
6	RMSP-1	+	+	+
7	RMSP-2	+	+	+
8	RMSB	+	+	+
9	<i>Pseudomonas putida</i> * (BR1)	+	+	+
10	<i>Bacillus subtilis</i> * (BR2)	+	+	+

Note: \* FR1 = Fungi Reference 1; \* FR2 = Fungi Reference 2; \*BR1 = Bacteria Reference 1; \*BR2 = Bacteria Reference 2; META- Malt Extract Tannic Acid; LNAM+MB- Low Nitrogen Agar Medium with Methylene Blue; LNAM+PR- Low Nitrogen Agar Medium with Phenol Red; + = Present ; - = Absent

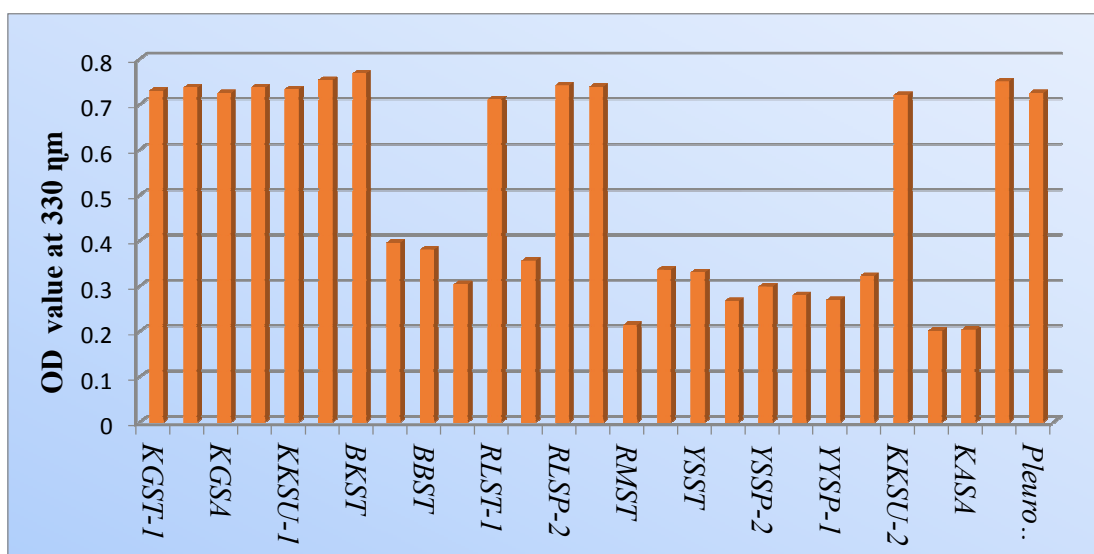
**Table 2. Dye decolorization by microorganisms**

Sl.No.	Fungal Isolates	PDA+ RBBR	PDA+ MG	PDA+ CR	PDA+ MB
1	KGST-1	+++	-	++	-
2	KGST-2	+++	-	+++	-
3	KGSA	++	++	-	-
4	KKST	+	+	-	-
5	KKSU-1	+	+	-	-
6	KKSP	+	-	++	-
7	BKST	+	-	+	-
8	RLST-1	-	-	-	-
9	RLSP-2	-	-	-	-
10	RMSP	++	++	-	-
11	KKSU-2	-	+	-	-
12	<i>Phanerochaete chrysosporium</i> *(FR1)	+++	-	+	-
13	<i>Pleurotus ostreatus</i> *(FR2)	++	+	-	-
Sl.No.	Bacterial Isolates	NA + RBBR	NA + MG	NA+ CR	NA + MB
1	KGSB-1	++	-	-	-
2	KGSP	+	-	-	-
3	KGSB-2	+	-	-	-
4	KKSB-2	-	-	-	-
5	BKSB	+	-	-	-
6	RMSP-1	+	-	-	-
7	RMSP-2	++	-	-	-
8	RMSB	+	-	-	-
9	<i>Pseudomonas putida</i> * (BR1)	+	+	-	+
10	<i>Bacillus subtilis</i> * (BR2)	+	+	+	-

Note: \* FR1 = Fungi Reference 1; \* FR2 = Fungi Reference 2; \*BR1 = Bacteria Reference 1; \*BR2 = Bacteria Reference 2; + = Positive for decolorization of dye; - = Negative for decolorization of dye



**Fig. 1. Preliminary screening of native bacterial isolates for enzyme activity by tannic acid method**



**Fig. 2. Preliminary screening of native fungal isolates for enzyme activity by tannic acid method**

the isolates tested positive for Lignin Peroxidase, but they were found to be less effective than reference fungi *Phanerochaete crysosporium*. The white-rot fungi produce extracellular enzymes that break the lignocellulolytic materials [27]. *Pleurotus ostreatus* produced more manganese peroxidase enzymes than other species. The *Pleurotus ostreatus* reference fungal strains were comparable to the native fungal isolates KGST-1, RLST-1, RLSP-2, RMSP, and KKSU-1. Manganese Peroxidase was non-detectable in few fungi, including KGST, KGSA, KKST, KKSU-2, and BKST. Several of the bacteria tested positive for Laccase enzyme activity. The fungal isolates, namely KGST-1, KGSA, KKST, KKSU-1, KKSP, and BKST were positive for Laccase synthesis, and these were superior to reference fungal stains. Laccase synthesis was absent in the fungal isolates KGST-2, RLST-1, RLSP-2, RMSP, and KKSU-2. All the three enzymes are responsible for lignin degradation; lack of any one of these enzymes will not complete the degradation process [28]. The white-rot fungi produce extracellular enzymes that breaks the lignocellulolytic materials.

#### 4. CONCLUSION

A variety of lignin-degrading fungi can be found in soil. The isolates had a strong ligninolytic activity in the current study. As a result, these isolates can be used to degrade complex organic material and create industrially essential

enzymes. Plant material can also be used as a low-cost raw material for the manufacture of enzymes because it gives nutrients to fungi.

#### DATA AVAILABILITY STATEMENT

Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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