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Isolation, Screening and Biochemical Characterization of Laccase Producing Bacteria for Degradation of lignin

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Abstract
The present work focuses on isolating, screening and biochemical characetization of laccase producing bacteria.Lignin is the most abundant aromatic polymer found as a major component of lignocellulose in plant cell walls and is extremely resistant to chemical and biochemical breakdown. Research on lignin biodegradation has accelerated greatly during the past 20 years, mainly because of the substantial potential applications of bio-ligninolytic systems in pulping, bleaching, converting lignins to useful products and treating of agricultural wastes using bacteria. Isolation and identification of environmental friendly bacteria for lignin degradation becomes an essential, because all the previous researches concentrated on using fungal treatments. However, bacteria seem to play a leading role in decomposing lignin because wood degrading bacteria have a wider tolerance of temperature, pH and oxygen limitations than fungi. Therefore, in this study soils were collected from wood industry and used for isolation of lignin degrading bacteria. Four bacterial strains were isolated by screening procedure based on their oxidative activity on ABTS (2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) and by a indicator Bromo phenol blue and later by the biochemical characterization strains were identified as Enterobacter, E.coli and two strains of Serritia.

Keywords: Lignin, Lignin degrading bacteria, Bio-ligninolytic system, ABTS,

INTRODUCTION

In industrialized countries like India, chemical contaminants of industrial effluent caused serious health problems. Major pollutants are supposed to be studied in more details such as lignin and its intermediates. Lignin is the most abundant aromatic, highly recalcitrant compound towards both chemical and biological degradation, and is second only to cellulose in its contribution to living terrestrial biomass (Crawford, 1981). Forest ecosystem contains about 1,50,000 million tons of wood, that possesses approximately 20-30% lignin. Thus the biodegradation of lignin play a crucial role in maintaining the carbon cycle in terrestrial ecosystems. But since the inception of research, biodegradation of lignin has been a challenge to researchers throughout the world.

To date, only a few groups of organisms are capable of degrading complex lignin polymers, and the white rot fungi best exemplify them. Most of the research concerning biodegradation of lignin has been centered on some fungi only such as Phanerochaete chrysosporium, Streptomyces viridosporus, Pleurotus eryngii etc.

Several developments in recent years have helped to make bioremediation feasible -the use of living organisms to decontaminate polluted soil or water. As we see due to these pollutants there are the significant risks of damage to the public health, we should take some action to diminish these risks utilizing the growing scientific knowledge. Biodegradation seems difficult to achieve the goal, due to lignin complex structure of phenylpropane subunits recalcitrant to normal hydrolytic treatments and because of the complexity of the enzymetic system involved. Lignin is a water insoluble polymer. Like other biopolymer, to be fully metabolized it must be broken down extracellulary into fragments that are small enough to enter cells. It has been the most poorly understood natural products. Due to its non-

phenolic aromatic nature, lignin units cannot be oxidized by lowredox-potential oxidoreductases, such as the plant peroxidases initiating the polymerization process. Metabolic studies with dimeric lignin model compounds have shown that some fungi and bacteria can cleave β-O-4 ether bonds, oxidatively cleave side chains between the α-β carbons and cleave alkyl-phenyl bonds. In fact, only a small group of highly specialized peroxidases secreted by ligninolytic fungi are able to degrade model compounds representing the main lignin substructures. The bulky nature of the heterogeneous lignin polymer forming a complex three-dimensional network represents an additional limitation for biodegradation since the enzyme accessibility is strongly reduced.

Moreover, using simple model compounds, it was possible to demonstrate that among the different oxidative enzymes produced by lignin-degrading organisms; only a group of basidiomycete haemperoxidases could directly attack the non-phenolic lignin network (Martínez, 2002; Hammel and Cullen, 2008). These enzymes include lignin peroxidase (LiP) initially described in *Phanerochaete chrysosporium*, the first basidiomycete whose genome was sequenced due to the interest on biological degradation of lignin (Martínez *et al.*, 2004), and a versatile peroxidase (VP) more recently reported in *Pleurotus* and *Bjerkandera* species, the former genus including species being able to degrade lignin selectively (Martínez *et al.*, 1999). VP is also able to oxidize Mn2+, as reported for *P.chrysosporium* manganese peroxidase (MnP). The Mn3+ resulting from the action of these two peroxidases oxidizes phenolic compounds but, in the presence of unsaturated lipids, it can also oxidize non-phenolic lignin via peroxidation radicals (Bao *et al.*, 1994). The molecular evolution of ligninolytic peroxidases has been shown by a recent study. Although significant advancement have been made in lignin research but all the basics and applied research work has centered on fungi only.

Besides wood-rot fungi, there are reports of bacteria that have the ability to break down lignin (Bugg et al., 2011a; Chen et al., 2012; Santhanam et al., 2011; Vicuna, 1988; Zimmermann, 1990). These lignin-degrading bacteria have mainly been isolated from soil and the guts of wood-eating insects, and belong to three classes, Actinomycetes, a-proteobacteria, and g-proteobacteria (Bugg et al., 2011a). In comparison to fungal lignin degrading enzymes, the bacterial enzymes implicated in lignin breakdown are much less well studied. It has been suggested that bacteria might use similar types of extracellular lignin-degrading enzymes to deconstruct lignin (McLeod et al., 2006; Ramachandra et al., 1988).

The goal of this research was to isolate novel lignin-degrading bacteria present in soil of wood industry. It is thought that the microorganisms in these soils have developed enzymatic systems for the rapid turnover of organic matter and that this process supports the rich diversity in this area (Parton et al., 2007). In this study, bacterial strains isolated from soil samples were screened based on their ABTS-oxidizing activity, and by Bromo phenol blue.

MATERIAL AND METHORDS:

Chemical required:

The chemicals and reagents used in this study were analytical grade. We need MSM Media (KNO_3 - 0.2%, K_2HPO_4- 0.1%, $MgSO_4$- 0.5%, NaCl- 0.5%, $FeSO_4$- 0.3%, NH_4NO_3- 0.5%, $CaCO_3$- 0.3%, $CuSO_4$- 0.1%, Glucose- 1%), NAM (Nutrient Agar Media), ABTS (2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate)), Bromo phenol blue, Tryptone, $CaCl_2$, Distilled water, Peptone, $NH_4H_2PO_4$, $Na_3C_6H_5O_7$, Bromothymol blue.

Collection of Soil and Isolation of Bacteria

Soil sample is collected from three different sampling sites of wood industry. The soil were collected from the surface to a depth using sterile spatulas and transported to the laboratory and stored at -20^{0}C.

Soil Characteristics:

Soil characteristics were determined using standard methods (APHA 1985). For each sampling site, three soil samples were combined and mixed together to construct a composite sample.

Bacterial Isolation and Screening:

For isolation of the soil bacteria, 250 gm soil sample was added to 250 ml of distilled water. The solution was stirred vigorously and then autoclaved at 15 psi for 45 min. Then collect the supernatant by centrifuging the soil sample and now supernatant called as soil extract. Prepare MSM (composition described above) in 100 ml soil extract and add 5 gm of soil in this medium. The medium was stirred vigorously and placed in incubator shaker for 2 days at 30^0C. Take 1 ml of sample from MSM and perform serial dilution in 0.9% NaCl. 100µl of the liquid mixture was serially diluted until a dilutution of 10^{11}. Then, 100µl of this solution from each dilution was plated on NAM. The plates are incubated at 30^0C for 2-3 days.

For the screening of isolated bacteria for the presence of lignin degrading enzyme (laccase activity) ABTS is used. Laccase activity was assayed at 30^0C using 2,2'- azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), a non-phenolic substrate that produces a green radical cation on oxidation by laccase. ABTS solutions of 2 mM were freshly prepared and then NAM plates were flooded with this solution. Colonies showing green color comform the presence of laccase activity.

Simultaneously, Bromo phenol blue is also used as a indicator for conforming lignin degrading activity. In order to study ligninolytic potential independently from lignin utilization, the decolourization of synthetic lignin-like dyes may be monitored. Dye decolourization was assessed in solid phase plate assays. Colonies were inspected for dye adsorption and cell-free incubations were assayed as control for abiotic dye decolourization. Bromo phenol blue show clear zone which indicate the presence of lignin degrading activity.

Biochemical Characterization:

To identify the selected stains biochemical characterization has been done. Different test are performed they include Indole test, Citrate test, Gram staining, Methyl red and Voges-Proskauer test.

RESULTS:

Naturally occurring microorganisms are having the ability to produce various enzymes. Now a day's most of the enzymes are important for human welfare and industry. In this study, the bacterial strains were isolated from the sample soil from wood industry because these industries are having lignin containg waste which was degraded by the native microbes that are growing over that waste. In such a way, it is fact that the microbes which are isolated from decayed wood soil may have ability to produce laccase enzyme.

For the screening has been done by ABTS and with the help of Bromo phenol blue dye as discussed above and the results was shown in plate 1 and 2. Colonies showing green color comform and clear zone in plates indicate the presence of lignin degrading activity or the presence of laccase activity. From the samples, many bacterial strains were isolated and screened; it was found some of those bacterial strains showed positive result for laccase production. The better zone and better color formed bacterial strain was considered for further study.

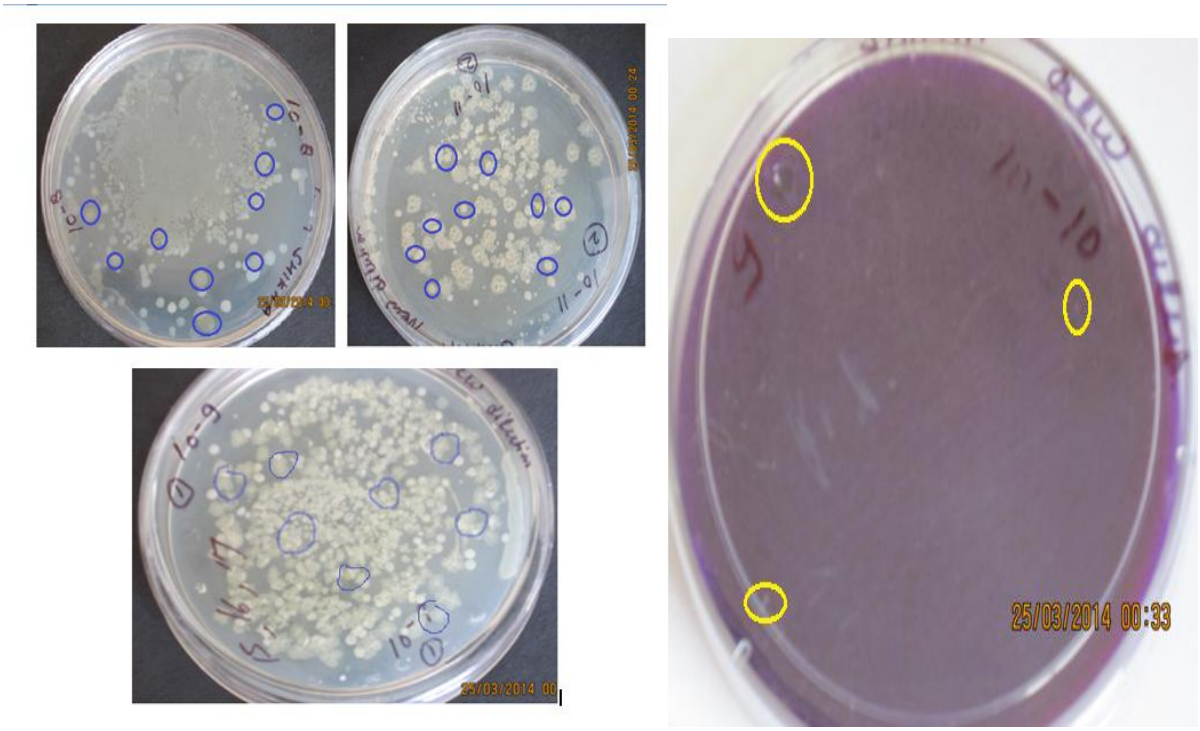

Plate1 and 2: Showing activity of bacterial isolates on NAM having ABTS andShowing activity of Bacterial Isolates on NAM having Bromo phenol blue .

For the identification of the selected lignin degrading stains, biochemical characterization has been done. Microscopic image of graom staining of selected staind and results of biochemical test are shown in Plate 3 and Table 1. And on the basis of these test the isolated strains are identified as Enterobacter, E.coli and two strains of Serritia.

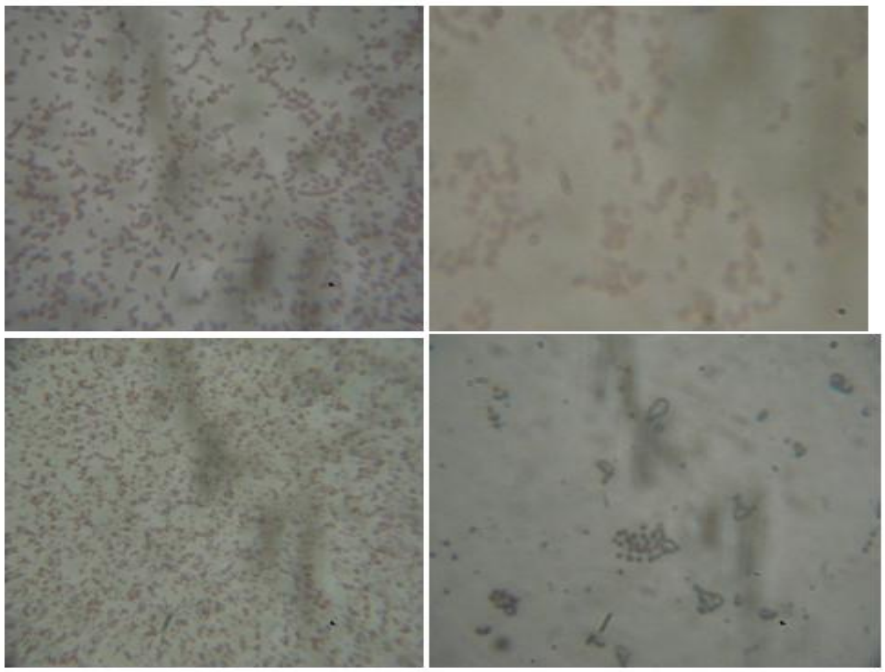

Plate 3: Showing Microscopic image of Gram staining of isolated bacterial strains.

Table 1: Result of Biochemical Characterization of selected bacterial strains.

Strain Name	Methyl red	Voges Test	Indole Test	Citrate Test	Gram Staining	Color	Bacterial Stain
A1	-ve	-ve	+ve	+ve	Gram –ve (Rod shaped)	Pink	Serratia sp.
A2	-ve	-ve	-ve	+ve	Gram –ve (Rod shaped)	Pink	Serratia sp.
A3	-ve	+ve	-ve	+ve	Gram –ve (Rod shaped)	White	Enterobacter
C1	+ve	+ve	+ve	-ve	Gram –ve (Rod shaped)	White	E.coli

CONCLUSION:

During the present study, we have isolated four lignin-degrading bacteria from soil samples of wood industry and identified these strains on the basis of their laccase activities on ABTS, decolourisation of Bromo Phenol Blue, an indicator dye and various biochemical assays. These types of microorganisms are probably rather common in the soil, and their role in the carbon circulation may be to degrade residual lignin. At least some of the microbes seem to use enzymes to degrade lignin. Such types of enzymes might be very interesting for technical applications, since they do not need cofactors, and may also have high specificity. In addition to laccase, it seems likely that there are additional enzymes and pathways of lignin degradation in these two bacteria yet to be identified.

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