Isolation and Characterization of Ligninolytic Bacteria from Bharda Khar Agro Field of Bhilai-Durg

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Abstract

Lignin is an organic substance binding the cells, fibres and vessels which constitute wood and the lignified elements of plants, as in straw. Lignin provides plants with compressive strength and protection from pathogens after cellulose; it is the most abundant renewable carbon source on Earth. The majority of plant biomass, including stems and leaves, is composed of lignocelluloses. Lignocelluloses are called "the second generation" feedstock for fuel and chemical production to emphasize the difference to the edible "first generation" feedstocks. Lignocelluloses are a complex and tightly organized matrix of three main polymers, cellulose, hemicelluloses and lignin. Historically, lignocelluloses recalcitrance has hindered its utilization as a feedstock in fuel and chemical production; however, the current drivers as well as technological development have renewed interest in lignocelluloses.

In present study isolation, identification and characterization of ligninolytic bacterial flora were done from the agro-fields, using a model industrial lignin residue from the Kraft process. 2 types of soil samples black and mixed soil were selected from agro fields of **Bharda Khar of Bhilai-Durg** for isolation of ligninolyitc bacterial colony. Microbes from both mixed and black soil samples were grown in solid media by Hungate method. The growing colonies then were counted and identified. The lignin degrader bacteria was selected qualitatively based on the diffusion zone diameter that formed around colony. Diffusion zone with colony size was used to determine the selected isolates. The pure cultures of lignin degrading microbes were selected and subjected to various morphological study, various types of differential staining and biochemical characterization tests to determine the identity of the bacteria isolates with reference to Bergey's Manual of Determinative Bacteriology. After screened and identification of lignin degrading bacterial strains were used for the check the quantitative estimation of lignin degradation capability. Presence of lignin degrading enzymes activity in lignin degrading bacteria were measured by Laccase activity, Manganese peroxidase (MnP), and Lignin peroxidase (LiP) activity.

Result of present studies shown that ten types of bacterial colonies (5-MS, 5-BS), were isolated from Bharda Khar agro field and used to check the activity of ligninolytic capability. Out of 10 colonies only **4** types of colonies (d, e-MS and B, C-BS), were shown potential of lignin degradation. The Morphological, gram's reaction, endspores staining reaction and biochemical characteristics of the isolates obtained from this agro-field soil samples identified with reference to Bergey's Manual of Determinative Bacteriology. These identified isolates include (d and B) - *Pseudomonas aerugionosa* and (e and C) - *Bacillus* species and both bacteria were shown presence of Laccase,

Manganese peroxidase (MnP) and Lignin peroxidase (LiP), lignin degrading enzymes. This result concluded that both kinds of strains were able to degrade lignin, which was second abundant and waste material in the world. It is also concluded that to expand on the range of products which can be obtained from lignocellulosic biomass, the lignin component should be utilized as feedstock for value-added chemicals such as substituted aromatics, instead of being incinerated for heat and energy. Enzymes could provide an effective means for lignin depolymerization into products of interest. In this study, soil bacteria were isolated by enrichment on Kraft lignin and evaluated for their ligninolytic potential as a source of novel enzymes. They may also have specific advantages for the depolymerization of the modified lignin residues typically encountered in waste streams from the pulping or 2nd generation biofuel/biobased chemicals industry.

KEYWORDS: Lignocellulosic, 2nd generation biofuel, Laccase, Manganese peroxidase (MnP), and Lignin peroxidase (LiP), *Pseudomonas* aerugionosa, Bacillus species

INTRODUCTION

Today the transport sector consumes more than half of the annually produced oil and only 2 % of the global fuel demand is met by refining renewable feed stocks into transportation fuels (International Energy Agency, 2012). Lignocelluloses are the most abundant renewable biomass resource on Earth and for the past 80 years it has been acknowledged as a potential feedstock for the production of fuels and chemicals (Himmel *et al.,* 2007). Presently, millions of tons of lignin and lignin-related compounds are produced as waste effluent from the pulping and paper industries. To date, less than 100 000 t a^{-1} of lignin obtained from the Kraft pulping process is commercially exploited (Foust *et al.,* 2008).

Lignin is a complex, three-dimensional aromatic polymer consisting of dimethoxylated, monomethoxylated and non-methoxylated phenylpropanoid subunits. It is found in the secondary cell wall of plants, where it fills the spaces between the cellulose, hemicelluloses and pectin components, making the cell wall more rigid and hydrophobic. After cellulose; it is the most abundant renewable carbon source on Earth. (Argyropoulos and Menachem, 1997). Such valorization would require controlled depolymerization of lignin, which is hampered by its high resistance towards chemical and biological degradation (Martinez *et al.,* 2005). Enzymes could provide a more specific and effective alternative for lignin depolymerization. Furthermore, biocatalytic processes generally take place under mild conditions, which lowers the energy input and reduces the environmental impact (Perez *et al.,* 2002; Sun and Cheng, 2002). Biodegradation of Lignin Wood-rotting basidiomycetous fungi that cause white rot in wood are the most efficient lignin degraders in nature (Kirk and Farrell, 1987; Eriksson *et al.,* 1990). These fungi produce an array of powerful ligninolytic enzymes such as laccases, lignin peroxidases (LiP's) and manganese peroxidases (MnP's) (Arantes and co-workers, 2007; Shary *et al.,*, 2008)

Ligninolytic bacteria are less well studied, but several examples have been found among Î±-proteobacteria (*e.g.*, *Sphingomonas* sp.[Wenzel *et al.,* 2002; Masai *et al.,* 2003; Masai *et al.,* 1999]), Î³-proteobacteria (*e.g.*, *Pseudomonas* sp. [Delalibera *et al.,* 2007])

and actinomycetes (*Rhodococcus*, Nocardia and *Streptomyces* sp. [Zimmermann, 1990; Bugg *et al.*, 2010]). The enzymes reported to be involved in bacterial lignin degradation are laccases, glutathione S-transferases, ring cleaving dioxygenases[Masai *et al.*, 2003; Allocati *et al.*, 2009], monooxygenases and phenol oxidases [Perestelo *et al.*, 1989]. Such enzymes are also involved in degradation of polycyclic aromatic hydrocarbons (PAHs), which show similar structural properties and resistance to microbial degradation as lignin [Allocati *et al.*, 2009; Canas *et al.*, 2007].

The enzymes are used for the degradation of many compounds, and it's used for biological functions such as textile, food, paper and pulp industries, organic, medical, pharmaceutical, and cosmetic and nanotechnology applications, and bioremediation too, having many functions in the microorganism. (Cullen 2002). They may also have specific advantages for the depolymerization of the modified lignin residues typically encountered in waste streams from the pulping or 2nd generation biofuel/biobased chemicals industry (International Energy Agency, 2012).

In the present study, the characterization and identification of naturally ligninolytic bacterial flora from the agro-fields, using a model industrial lignin residue from the Kraft process, which at present is the predominant process in the pulping industry.

MATERIALS AND METHODS

Sources of Isolates

The bacteria were isolated from 2 types of soil samples black and mixed soil of agro fields. The agro fields of **Bharda Khar of Bhilai-Durg** were selected for isolation of ligninolyitc bacterial colony.

Isolation and Selection of Ligninolytic Bacteria

Microbes from both mixed and black soil samples were grown in solid media by Hungate method **(Ogimoto and Imai, 1981).** In warm condition, media was divided into 3 tubes. Each selective substrate then dissolved, and then poured 15 ml each into Petri disc. Microbes sources soil sample were serially diluted with 10^{-5} dilution then (100µl) soil sample was inoculated for 7-14 days. The growing colonies then were counted and identified.

The lignin degrader bacteria was selected qualitatively based on the diffusion zone diameter that formed around colony **(Subbarao, 1993: Samingan, 1998: Martani, 2003)**. Each isolate was inoculated by spot method on nutrient agar that contains 1% tannic acid **(Subbarao, 1993)**. Diffusion and clear zone were measured after 7 days of anaerobic incubation. Diffusion zone with colony size was used to determine the selected isolates.

Identification of Selected Microbes

The pure cultures of lignin degrading microbes were selected and subjected to various morphological studies, various types of differential staining (Gram's and endospore) and biochemical characterization tests (catalase test, starch hydrolysis, Indole, MR-VP, simmon's citrate agar, fermentation, H_2S production, nitrate reduction, urease, casein hydrolysis, gelatin hydrolysis) to determine the identity of the bacterial isolates with reference to Bergey's Manual of Determinative Bacteriology **(Buchanan and Gibbon, 1974).**

Lignin Degradation Study in Pure Culture

After screening and identification of lignin degrading bacterial strains of **Bharda Khar of Bhilai-Durg** argofield were used for the check the quantitative estimation of lignin degradation capability by **Chandara *et al.*, (2007)** and For the measurement of lignin degradation, 1 ml of samples were centrifuged at 15000 rpm for 5 min. Supernatant (250µl) was diluted by adding 2.5 ml phosphate buffer (pH 7.6) and absorbance measured at 280 nm for lignin degradation on a UV-visible spectrophotometer (Perkin Elmer Lambda EZ201 UV/VIS Spectrometer) **Lara *et al.*, (2003)** methods.

Enzyme Assays

Presence of lignin degrading enzymes activity in lignin degrading bacteria were measured by followed-

- Laccase activity by **(Machado and Matheus, 2006)** method
- Manganese peroxidase (MnP), by **(Glenn and coworkers, 1986)** method and
- Lignin peroxidase (LiP) activity by **(Tie *et al.*, 1988)** method.

RESULTS AND DISCUSSIONS

In the present study, the isolation and characterization of naturally ligninolytic bacterial flora from **Bharda Khar** agro-fields **of Bhilai-Durg** were done. The bacteria were isolated from 2 types of soil samples black and mixed soil of agro fields.

Isolated Bacterial Colonies from Bharda Khar Agro Fields

Bacterial colonies were isolated from both mixed and black soil samples of Bharda Khar airfield and grown in solid media by Hungate method (Ogimoto and Imai, 1981). **10** types of bacterial colonies (5-MS, 5-BS), were isolated and used to check the activity of ligninolytic capability Bharda Khar agro field (Table- I).

Qualitative Selection of Ligninolytic Bacterial Colonies

All ten isolated bacterial colonies of Bharda Khar were subjected for qualitative selection of the lignin degrading bacteria. Selection was based on the diffusion zone diameter that formed around colony **(Subbarao, 1993: Samingan, 1998: Martani, 2003)**. Each isolate was inoculated by spot method on nutrient agar that contains 1% tannic acid **(Subbarao, 1993)**. Diffusion and clear zone were measured after 7 days of anaerobic incubation. In Bharda Khar agro field out of 10 colonies only **4** types of colonies (2-MS, 2-BS), were shown diffusion zone with colony size was determined the potential of lignin degradation (Table-II).

Identification of Selected Ligninolytic Bacterial Colonies

In present studies Bharda Khar agro field **4** bacterial colonies (d, e-MS and B, C-BS), were shown potential of lignin degradation. The Morphological characteristics of the obtained from the soil samples on Nutrient Agar (NA) and Eosin Methylene blue (EMB) agar was shown in (Table-III). The gram's reaction and endspores staining reaction for the characterization of isolates obtained was also shown on Table-III. The Biochemical characteristics of the isolates obtained from this agro-field soil samples was shown in Table-IV. The isolated bacteria species were identified with reference to Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). These identified isolates included (d and B) - *Pseudomonas aerugionosa* and (e and C)- *Bacillus* species (Table IV).

Detection of Lignin Degrading Enzyme

Present studies of lignin degrading bacteria (d and B) - *Pseudomonas aerugionosa*, (e and C) - *Bacillus* species shown that in both bacteria Laccase, Manganese peroxidase (MnP) and Lignin peroxidase (LiP) lignin degrading enzymatic activity were positive (Table- V).

Odier and co-workers (1981) were selected eleven gram-negative aerobic bacteria (*Pseudomonadaceae* and *Neisseriaceae*) out of 122 soil isolates for their ability to assimilate poplar dioxane lignin without a cosubstrate. **Zhang *et al.*, (2002)** researched enzymes production, the degradation rate of lignin and strain growth of alkaliphilic ligninolytic bacteria strain No. 6 in alkaline liquid medium (pH approximately 10.5) with compounded carbons. The results showed that the highest activities of laccase and MnP were 2915.37 U/L (4th d) and 1152.88 U/L (8th d), respectively, and the strain degraded 49.84% lignin of wheat straw during ten days cultivation. **Ahmad, *et al.*, (2010)** have been developed two spectrophotometric assays to monitor breakdown of the lignin component of plant lignocelluloses. Two soil bacteria known to act as aromatic degraders, *Pseudomonas putida* and *Rhodococcus* sp. RHA1, consistently showed activity in these assays, and these strains were shown in a small scale experiment to breakdown lignocellulose, producing a number of monocyclic phenolic products. **Bandounas, *et al.*, (2011)** studied on the base of 16S rRNA gene sequencing and phenotypic characterization; the organisms were identified as *Pandoraea norimbergensis*

LD001, *Pseudomonas* sp LD002 and *Bacillus* sp LD003. The ligninolytic capability of each of these isolates was assessed by growth on high-molecular weight and low-molecular weight lignin fractions, utilization of lignin-associated aromatic monomers and degradation of ligninolytic indicator dyes. *Pandoraea norimbergensis* LD001 and *Pseudomonas* sp. LD002 exhibited best growth on lignin fractions. **Rodriguez *et al.*, (2011)** research was characterized and identified bacterial strains with ligninolytic activity. 150 different strains were isolated from compost, cow dung, and straw; they were grown on selective media containing commercial fiber, straw, or commercial lignin. 80 strains showed ligninolytic activity. From these, three strains (34, 40, and 30) showed the most activity for Li peroxidase, greater activity for Mn peroxidase and laccase. Morphological, biochemical, and DNA analysis indicate that at least two of the best ligninolytic strains are *Bacillus* spp. **Gong *et al.*, (2013)** reported two bacterial strains CGM123 and GWD275, *Filimonas lacunae* (93.03%) and *Bacillus methylotrophicus* (99.86%), isolated from forest soil, showed highest potential abilities to degrade cellulose, xylan and lignin. **Rahman *et al.*, (2013)** were isolated three aerobic SHC1, SHC2, and SHC3 as *Bacillus* sp., *Ochrobactrum* sp., and *Leucobacter* sp., respectively with 99% sequence similarity lignin-degrading bacterial strains from palm oil plantation soils. Bacillus sp. SHC1 produced the highest manganese peroxidase (MnP) of 2313.4 U/L on the third day and the highest lignin peroxidase (LiP) of 209.30 U/L on the fifth day of fermentation. The optimum pH and temperature for the production of ligninolytic enzymes by *Bacillus* sp. SHC1 were pH 8 and 30 °C.

Previous studied were shown that *Pseudomonas aerugionosa* and *Bacillus* sp. have highest degrading capability of lignin and also present all 3 enzymatic activities [Laccase, Manganese peroxidase (MnP) and Lignin peroxidase (LiP)] and their highest enzymatic activity found in *Bacillus* sp. Present study shown similarity with previous research work. The bacterial isolates in this study appear to have an alternative type of ligninolytic system. The enzymes are presumably cell-surface associated, in view of the large size of lignin, whereas fungal lignin degradation occurs via extracellular enzymes and secreted secondary metabolites. Thus, a new and presumably vast source may be tapped for novel ligninolytic enzyme activities. A few considerations, however, must be taken into account when hunting for novel ligninolytic activities for lignin valorization.

CONCLUSION

In the present study, the characterization and identification of naturally ligninolytic bacterial flora from the **Bharda Khar** agro-fields of **Bhilai-Durg**, using an industrial lignin residue from the Kraft process. The bacteria were isolated from 2 types of soil samples black and mixed soil sample of agro fields. In present investigation found that from the both soil samples of Bharda Khar agro-field *Pseudomonas aerugionosa* and *Baccillus* sp., 2 types of ligninolytic bacterial colonies were isolated. Both type strains given positive results for various ligninolytic enzymes *i.e.* (Laccase, Magnese Peroxidase and Lignin Peroxidase). This result concluded that both kinds of strains were able to degrade lignin substrate which was second abundant and waste material in the world. It is also concluded that to expand on the range of products which can be obtained from lignocellulosic biomass, the lignin component should be utilized as feedstock for value-

added chemicals such as substituted aromatics, instead of being incinerated for heat and energy. Enzymes could provide an effective means for lignin depolymerization into products of interest. In this study, soil bacteria were isolated by enrichment on Kraft lignin and evaluated for their ligninolytic potential as a source of novel enzymes. They may also have specific advantages for the depolymerization of the modified lignin residues typically encountered in waste streams from the pulping or 2nd generation biofuel/biobased chemicals industry.

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TABLE-I: BACTERIAL COLONIES ISOLATED FROM BHARDA KHAR AGRO-FIELDS

Sample Site	**F1**	
Types of Soils	**MS**	**BS**
Types of Colonies	**5**	**5**
No. of Colonies	**a- 5** **b- 36** **c- 64** **d- 35** **e- 5**	**A-5** **B-36** **C-64** **D- 35** **E- 5**

Note: MS- Mixed Soil, BS- Black Soil, UN- Uncountable, F1-Bharda Khar

TABLE-II: LIGNINOLYTIC COLONIES SELECTED FROM BHARDA KHAR AGRO-FIELDS

Sample Site	F1	
Types of Soils	MS	BS
Types of Colonies	2	2
Selected ligninolytic colonies and their Zone Diameters (mm)	30mm-d 28mm-e	30mm-B 28mm-C

TABLE-III: SHOWN MORPHOLOGICAL CHARACTERISTIC OF ISOLATED MICROBES FROM BHARDA KHAR AGRO-FIELDS

Isolates	Morphological Characteristics	Organims
d, B	Non-spore forming, Gram negative short rods, colourless colony on Nutrient Agar, Brown at 4 and 42^{O}C	*Pseudomonas aerugionosa*
e, C	Spore forming, Gram positive rods, creamy white colony on Nutrient Agar entire margin	*Bacillus* sp.

TABLE-IV: SHOWN BIOCHEMICAL TEST FOR IDENTIFICATION OF ISOLATED BACTERIA BHARDA KHAR AGRO-FIELDS

S. No.	Biochemical test	Bharda Khar Agro Field	
		MS-d, B Bacterial colonies	BS-e, C Bacterial colonies
1.	Motility test	+	+
2.	Catalase test	+	-
3.	6.5% NaCl	-	ND
4.	Glucose fermentation test	A/G	A/G
5.	Lactose fermentation test	A	A/G
6.	Sucrose fermentation test	A/G	A/G
7.	Starch Hydrolysis test	-	-
8.	Indole test	-	-
9.	MR Test	-	-
10.	VP Test	+	+
11.	Citrate test	-	+
12.	Urease test	-	-
13.	Gelatin Hydrolysis Test	+	+
14.	H2S Production Test	+	+
15.	Nitrate Utilization Test	+	-

17.	**Lipid Hydrolysis Test**	+	+
18.	**Oxidase Test**	+	+

Note: ND-Not Determined, A -Acid, A/G-Acid/Gas, + =Positive, - =Negative, (+) =Late Positive

TABLE-V: SHOWN LIGNIN DEGRADATION STUDY OF ISOLATED BACTERIA FROM BHARDA KHAR AGRO-FIELDS

S. No.	Lignin Degrading Bacterial colonies	Presence of Lignin Degrading Enzymes		
		Laccase	Manganese peroxidase	Lignin peroxidase (LiP)
1.	***Pseudomonas aerugionosa***	+	+	+
2.	***Bacillus* sp.**	+	+	+