

Original Research Article

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## Isolation and Screening of Lignin Degrading Bacteria from Different Natural and Organic Sources

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### ABSTRACT

Increased consumption and high dependence on non – renewable sources has substantially contributed to global warming and environmental pollution. Hence, alternative strategies for energy production have to be looked and one of the alternative is producing 2<sup>nd</sup> generation Bio-ethanol from lingo-cellulosic material. Maize cob is one of the Lignocellulosic material is one the substrate, which can be used for Bio-ethanol production (It is rind after removing the seeds). The present study is focused on to look for novel strains of lignin degrading bacteria from various organic sources. The different sample sources used for the isolation of lignin degrading bacteria were from cow dung, compost, forest soil, garden soil in agriculture college, Hassan. Enrichment of sample source was also done by mixing the paper, corncob, compost, cowdung and soil in different proportions and kept for 15 days at room temperature. Enriched samples were also used as a source for isolation of lignin degraders. Totally 64 colonies were isolated by screening in 7 different sample sources, from those 64 isolates, only 25 isolates confirmed as lignin degraders through conformational test. Decolourization zone produced by 25 isolates was measured. Eight isolates that produced large decolorization zone (0.5 to 1 cm) were selected and further screened for their tolerance to lignin by culturing them on 2% lignin. The results pertaining to growth on 2% Alkaline lignin is, isolate CC- 10 was grown within 24hr followed by CM-3, PC- 2, PC- 3, PC-5 and CC- 8 they were grown within 48hr. The growth of FS- 3 and CC-10 were seen within 72hr. Based on the results obtained the slow grower FS-3 and CC-10 was discarded. Six potential isolates which are showing large clear zone (decolourized) of methylene blue dye and also rapid growth on MSM with 2% alkaline lignin were selected. Based on this to reconfirm the isolates as a lignin degrading bacteria Protein profiling of the six isolates has been done by using SDS – PAGE technique.

#### Keywords

Maizecob, degradation, Lignin degrading bacteria, SDS – PAGE, Laccase

#### Article Info

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## Introduction

India is sixth in the world energy need and overall demand of crude oil is raised by 5.6% until 2011. The scenario of rising and volatile prices in world oil market and respective foreign exchange cost are the main risk factors for Indian economy and social development prospects. Further, increased consumption and high dependence on non – renewable sources has substantially contributed to global warming and environmental pollution. Hence, an alternative strategy for energy production has to be looked and one of the alternative is producing 2<sup>nd</sup> generation Bio-ethanol from lignocellulosic material. In Karnataka State, maize is grown in an area of 12.43 lakh hectares with an annual production of 30.15 lakh tonnes, out of this around 20 lakh tonnes of maize cobs is produced after the processing (Project director review, 2013, AICRP on Maize). The residue left over after harvesting of maize is the thrash and the cobs (It is rind after removing the seeds). The thrash is directly used as feed to the farm animals and cobs are very rigid in nature because of high lignocelluloses and presently the farmers are mainly using maize cobs as a fuel purpose (Direct burning for cooking purpose) which releases lot of Carbon materials to the environment and create environmental pollution. Lignin is the most structurally complex, possessing a high molecular weight and the most recalcitrant, consisting of various biologically stable linkages (Perez *et al.*, 2001). The plant cell wall consists of cellulose, hemicelluloses, pectic substances and lignin. After cellulose, lignin is the second most abundant renewable biopolymer in the biosphere (Rahman *et al.*, 2013). The breaking of lignocellulosic material is very difficult due to its complex structure (Himmel *et al.*, 2007), it has to be broken down to release fermentable sugars which can be utilized further for Bio ethanol production. There are various physical, chemical and biological

methods have been developed to degrade lignocellulosic material, which are expensive and energy consuming. The present study is focused on to look for novel strains of lignin degrading bacteria from various organic sources, which can be a cost effective method to degrade maize cob and further used for the production of bio-ethanol production.

## Materials and Methods

The study was executed at the College of agriculture, Hassan (University of Agricultural Sciences, Bangalore), Karnataka.

### Isolation of bacteria from different organic sources

The different sample sources used for the isolation of lignin degrading bacteria were from cow dung, compost, forest soil, garden soil in college of agriculture, Hassan. Enrichment of sample source was done by mixing the paper, corncob, compost, cowdung and soil in a different ratios and kept for 15 days at room temperature [Compost+ Soil+ Paper (4:2:1), Compost+Soil+Maize cob (4:2:1), Compost+Soil+Cowdung (2:2:1)]. Enriched samples were also used as a source for isolation of lignin degraders (Table 1).

The specific media used for isolation is minimum salt medium agar containing alkaline lignin (MSML-agar). Media Composition is K<sub>2</sub>HPO<sub>4</sub>, 4.55g; NH<sub>4</sub>NO<sub>3</sub>, 5g; H<sub>3</sub>BO<sub>3</sub>, 0.5g; CaCl<sub>2</sub>, 0.01g; KH<sub>2</sub>PO<sub>4</sub>, 0.53g; Trace element solution 1 ml (ZnSO<sub>4</sub>.7H<sub>2</sub>O, 2.2g; Mn acetate, 0.5g; FeCl<sub>3</sub>, 0.5g; CuSO<sub>4</sub>.6H<sub>2</sub>O, 0.16g; Molybdic acid, 0.11g; Na<sub>2</sub> EDTA, 5g; and distilled water, 1000 ml) and distilled water 1000ml. Isolation was done using the procedure followed by Sasikumar *et al.*, (2014).

MSML media consisted of 1% alkaline lignin is used for isolation of bacteria. Alkaline

lignin was prepared by using maize cob substrate. With 6 g maize cob powder, 40 ml of 1% H<sub>2</sub>SO<sub>4</sub> was added and heated in hot air oven at 80°C for 20 min. Allowed to cool, followed by 150 ml of 4 % NaOH was added and boiled for 30 min. The dark brown coloured alkali lignin was filtered and autoclaved at 15 lbs for 15 min. Inoculation was done by placing 5 g sample in 100 ml broth and incubated at 120 rpm for 7 days at 38.5°C temperature. Enriched sample of 1 ml were transferred to 99 ml of sterile NaCl, this solution was stirred vigorously and allowed to settle down. Using 1 ml of the liquid mixture, serial dilution was performed up-to 10<sup>-7</sup>. From each dilution about 100µl of sample was spread on plate containing MSML agar. Plates were incubated at 30°C for 7 days until colonies were developed. Isolated colonies were streaked onto fresh MSML agar plates repeatedly to obtain pure culture.

### **Confirmation test for screening lignolytic activity**

According to Sasikumar *et al.*, (2014), isolated colonies were screened by using methylene blue dye as an indicator. Pure cultures were streaked on methylene blue containing (0.25 g/L) containing LB agar plate. Plates were incubated at 30°C for 72 hr. The microbes possess lignolytic enzymes undergoes oxidation of indicator dye resulting in decolourisation of methylene blue. The decolourised colonies were selected as lignin degraders. The selected isolates were maintained through sub-culturing and also maintained at 4°C temperature.

### **Screening of the microbial isolates on 2% alkaline lignin**

The eight isolates further screened to know the potentiality of utilizing 2% Alkaline lignin as a sole carbon source and grow. The Microbial isolates were plated on Minimal media

containing 2% Alkaline lignin and observed the growth over a period of 72 hours (Chandra *et.al.*, 2010).

Eight isolates showing large decolourization zone (0.5 to 1 cm), were inoculated in MSM agar plate amended with 2% alkaline lignin. Growth was observed daily. On the basis of growth rate, isolates were distinguished as, very fast grown isolates which were grown within 24 hours, fast grown isolates grown within 48 hours and slow grown isolate grown after 48 hours. Among eight isolates, one isolate (CC-10) was grown within 24 hours, five isolates (CM-3, PC-2, PC-3, PC-5 and CC-8) were grown within 48 hours and two isolates (FS-3, CC-9) were grown after 48 hours. Six isolates showing rapid growth (CC-10, CM-3, PC-2, PC-3, PC-5 and CC-8) on MSM agar with 2% alkaline lignin was selected (Table 2) as potential isolates to degrade lignocellulosic biomass.

### **Protein profiling of the screened microbial isolates**

SDS PAGE was done to check whether selected isolates are producing laccase enzyme. Bands were compared with three standard protein bands BSA (Bovine serum albumin), Amylase and Protinase of band size of 66, 54 and 28 kDa respectively. Molecular size of sample proteins was determined using Image J software. Protein bands produced by isolates from both minimal media without cob and minimal media with cob were compared and it was observed that some isolates showed large size of protein bands isolated from media with cob compared to media without cob. So the isolates which were producing extra proteins in media with cob may considered as lignocellulose degrading enzymes and that isolates were further checked for their capacity to produce reducing sugars and phenolics by degrading maize cob. By analysing all the three parameters (protein,

reducing sugars and phenolics), finally three efficient isolates (PC-5, CM-3, PC-2) were selected for maize cob hydrolysis.

Protein profiling is done by using SDS PAGE to check whether selected isolates are producing laccase enzyme. Bands were calibrated with three standard protein *viz.* bovine serum albumin 64 KDa), amylase (54 KDa) and Protease (24 KDa). Molecular size of sample proteins were calculated using Image J software developed at the National Institutes of Health and the Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin). Further the microbial isolates were grown in two conditions; one with Nutrient Media (where all the nutrients are available for the growth) and another minimal media with maize cob as sole carbon source.

#### **Protein extraction (Bhaduri and Demchick, 1983)**

Isolates were inoculated into both LB broth and minimal media containing 1 g maize cob powder, allowed to growth for 24 hour and stored in a refrigerator. 2 ml of broth culture was taken in microfuge tube and then centrifuged at 7000g for 15 minutes. The supernatant was discarded, pellet was washed with 0.9% saline twice. The cells were then suspended in 2 ml of acetone (Ice cold) and allowed to stand on ice for 5 min and collected by centrifugation. Then cells were suspended in 1.5 ml of 1 % SDS and disrupted using sonicator. This whole total cell protein extract was used for protein estimation and profiling by Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS PAGE)

#### **SDS-Poly Acrylamide Gel Electrophoresis (SDS PAGE)**

Vertical slab gel electrophoresis unit was set to run SDS PAGE. 10 % Resolving gel (water,

19.8ml; 30% Acrylamide:Bisacrylamide, 16.7 ml; 1.5 M Tris (pH 8.8), 12.5 ml; 10 % SDS, 0.5ml; 10%APS, 0.5ml; TEMED, 20  $\mu$ l) was poured up-to 3/4 level between the glass plates and overlay with isopropanol for normalization of the gel edges. After polymerization isopropanol was removed and washed with double distilled water. Comb was placed and 5% stacking gel (water, 6.8ml; 30% Acrylamide:Bisacrylamide, 1.7 ml; 1 M Tris (pH 6.8), 1.25 ml; 10 % SDS, 0.1ml; 10%APS, 0.1ml; TEMED, 10  $\mu$ l) was poured and allowed to polymerize, then comb was removed without distorting the shapes of the well. Carefully gel was installed into the electrophoresis unit with the reservoir buffer (1M, Tris glycine buffer, pH 8.5). Samples were prepared by mixing it with loading dye in the proportion of 45  $\mu$ l sample and 15  $\mu$ l loading dye (60mM Tris HCL (pH 6.8), 0.6ml, 2.5% SDS, 2.5ml; glycerol, 1ml;  $\beta$  Mercaptoethanol, 250  $\mu$ l; 0.002% Bromo phenol blue, 20  $\mu$ l) and then placed it in a boiling water bath for 5-10 minutes for destabilization and incorporating the uniform charges to the protein for electrophoresis. Samples and standard proteins were loaded into well and connected the unit to electrical power pack and run at 120V for 6 hours (Kurien and Scofield, 2012).

#### **Staining and Destaining (Kurien and Scofield, 2012)**

After electrophoresis, gel was removed from glass plates and transferred to staining tray and rinse in a fixing solution (Fixing solution (100 ml): Glacial Acetic Acid, 10 ml; Methanol, 50 ml; Water: 40 ml) for about 10 minutes. Then poured off the fixing solution. Rinsed with water for 30 seconds. Then added 150 ml of staining solution (Staining solution(100 ml): Coomassie brilliant blue R-250, 0.25 g; Glacial Acetic Acid, 10 ml; Methanol, 50 ml; Water: 40 ml), kept in microwave for 50 to 60 seconds until the

solution just starts to boil. Incubate at room temp with gentle shaking for 2-5 min. Heating allows the gel to stain faster. Poured off the staining solution and then repeated the process with another fresh 150 ml of staining solution. Poured off the staining solution and then rinsed the gel with water for 15 seconds. Then added 150 ml of destaining solution (Destaining solution (200 ml): Glacial Acetic Acid, 20 ml; Methanol, 100 ml; Water, 80 ml), kept in microwave for 40 to 50 seconds, kept in gentle shaker for 10 minutes at room temperature. Poured off the destaining solution and repeated the cycle with fresh destaining solution for 3 to 4 times. Blue stained bands on the gel were observed and image was captured under gel documentation unit.

### Results and Discussion

The different sample sources used for the isolation of lignin degrading bacteria and total number of colonies screened and confirmed isolates from each source are tabulated in table 1. Totally 64 colonies were isolated by screening in 7 different sample sources, from those 64 isolates, only 25 isolates confirmed as lignin degraders through conformational test (Table 2 and Figure 1). The decolourization of methylene blue has been used previously as an indicator of lignin

peroxidase enzyme activity (Ferrer'ra – leitao *et al.*, 2007), which is one of the enzyme responsible for lignin degradation. Maximum of 11 isolates were isolated from maize cob enriched sample only, 5 isolates from paper enriched sample, 4 from compost and cowdung enriched sample. From forest soil, 3 isolates were showed positive, while from garden soil and cowdung, one isolate each showed positive in conformational test. Decolourization zone produced by 25 isolates was measured and presented in Table 2.

Eight isolates that produced large decolorization zone (0.5 to 1 cm) were selected and further screened for their tolerance to lignin by culturing them on 2% lignin.

The results pertaining to growth on 2% Alkaline lignin is presented here CC- 10 was grown within 24hr followed by CM-3, PC-2, PC- 3, PC-5 and CC- 8 they were grown within 48hr. The growth of FS- 3 and CC-10 were seen within 72hr. Based on the results obtained the slow grower FS-3 and CC-10 were discarded (Table 3). The capability of soil isolated bacteria to degrade efficiently and assimilated lignin as a sole carbon sources agreed with findings of Morrii *et., al.*, (1995) and Hassan and Hanafy, (2009) (Fig. 2 and 3).

**Table.1** Total number of colonies screened and confirmed isolate

Sl.No.	Sample source	Number of colonies screened	Number of Confirmed isolates
1	Cowdung	7	1
2	Compost	7	0
3	Compost+Soil+Paper (4:2:1)	8	5
4	Compost+Soil+Maize cob (4:2:1)	13	11
5	Compost+Soil+Cowdung (2:2:1)	9	4
6	Forest Soil	9	3
7	Garden Soil	11	1
<b>Total</b>		64	25

**Table.2** Screening of confirmed colonies using decolourization of methylene blue in LB media

Sl.No	Isolate No.	Zone of decolourization(cm)
1	CM-3	1
2	CC-8	1
3	PC-5	0.8
4	FS-3	0.6
5	PC-2	0.6
6	PC-3	0.5
7	CC-9	0.5
8	CC-10	0.5
9	CC-4	0.4
10	CM-2	0.3
11	CM-4	0.3
12	PC-1	0.3
13	CC-5	0.2
14	GS-1	0.1
15	CC-1	0.1
16	CC-3	0.1
17	C-11	0.1
18	CC-7	0.08
19	CW-1	0.05
20	FS-2	0.05
21	PC-4	0.05
22	CC-2	0.05
23	CC-6	0.04
24	FS-1	0.03

**Table.3** Isolates grown on 2% Alkaline lignin for measurement of their tolerance

Sl.No	Isolate no.	Growth rate
1	FS-3	+
2	CM-3	++
3	PC-2	++
4	PC-3	++
5	PC-5	++
6	CC-8	++
7	CC-9	+
8	CC-10	+++

+++; very fast growth(within 24 hr)

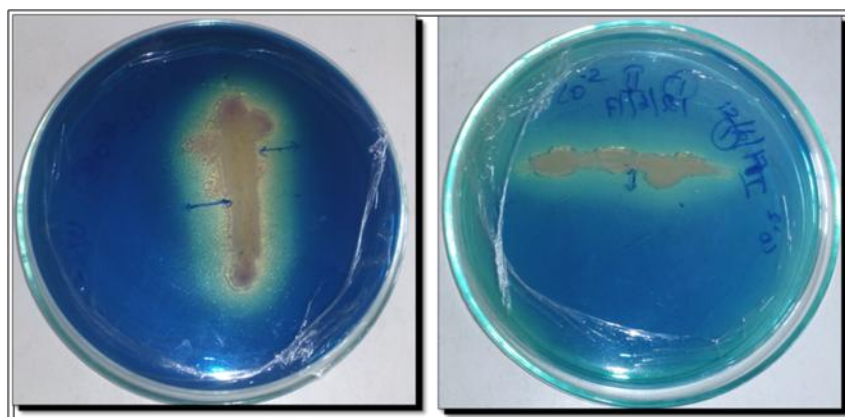
++; fast growth(within 48 hr), +; slow growth(within 72 hr)



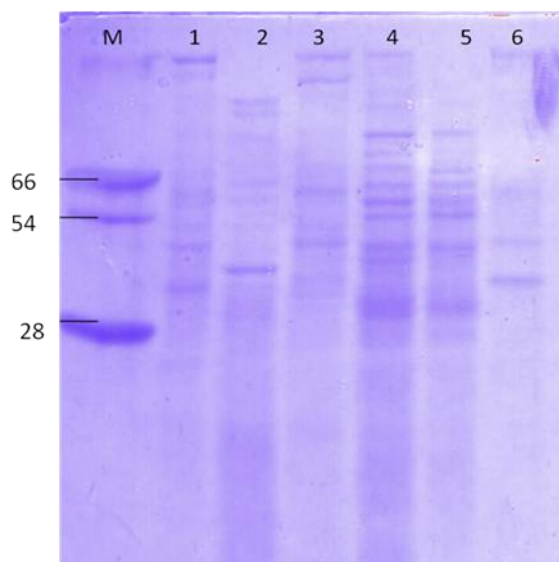
**Table.4** Molecular weight of proteins produced by isolates grown both in Nutrient broth without cob and Minimal media containing cob

Sl. No	Isolate No	Minimal media with cob (kDa)
1	<b>PC-5</b>	<b>96,92,87,78,67,62,53,50,38,26,17</b>
2	CC-8	84,82,79,74,66,63,58,51,43,36,20
3	<b>CM-3</b>	<b>97,91,86,79,68,62,54,50,41,36,3</b>
4	<b>PC-2</b>	<b>97,93,85,76,69,66,64,60,57,48,45,42,30,27</b>
5	PC-3	78,70,67,65,62,57,45,35,29
6	<b>CC-10</b>	<b>97,93,63,50,40</b>

**Figure.1** Decolourization of methylene blue in LB media by isolates

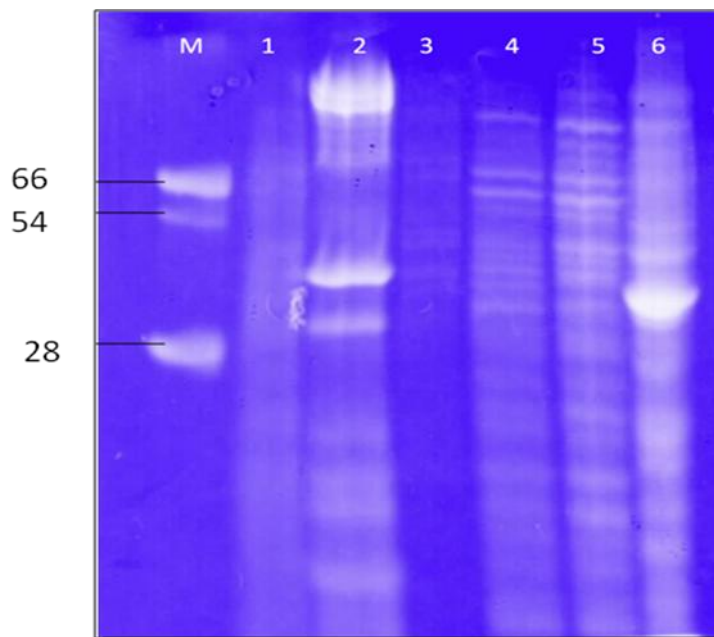


**Figure.2** Protein profile of the six isolates grown in minimal media with cob



(M: Molecular weight standard 66, 54 and 28 kDa; 1: PC-5; 2: CC-8; 3: CM-3; 4: PC-2; 5: PC-3; 6: CC-10.)

**Figure.3** Protein profile of the six isolates grown in nutrient broth without cob



(M: Molecular weight standard 66, 54 and 28 kDa; 1: PC-5; 2: CC-8; 3: CM-3; 4: PC-2; 5: PC-3; 6: CC-10.)

Six potential isolates which are showing large clear zone (decolourized) of methylene blue dye and also rapid growth on MSM with 2% alkaline lignin were selected. Further these 6 isolates can be used in studies related to biodegradation of maize cob (Table 4).

To degrade the lignin, organisms has to produce extracellular enzymes, namely Laccases, Lignin peroxidises (LiPs), Manganese peroxidises (MnPs) and Versalite peroxidises (Buggy *et al.*, 2011; Hatakka, 1994). Based on this to reconfirm the isolates as a ligning degrading bacteria Protein profiling of the six isolates has been done by using SDS – PAGE technique to check the organisms is producing Laccases enzyme as an indicator. Protein bands produced by isolates grown in both minimal media without cob and minimal media with cob were compared and it was observed that some isolates showed expression of a certain prominent bands as an indication of differential gene expression. Therefore, the isolates grown in minimal media with maize cob and producing extra proteins may be

considered as lignocellulose degrading enzymes (Ahmad *et al.*, 2011), finally three efficient isolates (PC-5, CM-3, PC-2) were selected for maize cob hydrolysis.

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