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SCREENING AND ISOLATION OF LIGNOCELLULOSE DEGRADING BACTERIA FROM SOILS OF RAIGAD DISTRICT

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Abstract:

India is an agriculturist nation leading to generate huge amount of lignocellulosicbiomasses from plant and agricultural wastes. Lignocellulosic biomasses abundant source of renewable value added byproducts needs degradation using microorganisms to overcome lignocellulosic deposition and environmental problems. Considering this, the rainforest soils ofRaigad district was assessed for isolation of lignin degrading bacteria. Lignin containing minimal salt media was used to isolate bacterial isolatesfrom the soil samples. Preliminary screening of lignin degrading bacteria was carried out by testing against methylene blue indicator dye containing LB medium. Fourteen bacterial isolates showed positive results and subsequently assessed for ability to degrade lignin. Based on the morphological and biochemical analysis the isolates were identified as *Pseudomonas* sp. *Bacillus* sp. The isolated bacterial flora has significant potential applications for the treatment of lignin degradation and lignin related environment pollutants.

Keywords: Lignin, lignin degrading bacteria, MSM-Lignin medium, methylene blue dye, *Pseudomonas* sp., *Bacillus* sp.

1. Introduction:

Lignin forms tight association with cellulose and hemicelluloses to form lignocelluloses as a rigid recalcitrant material in woody plants (Lee et al., 2014). Lignin is the second most abundant biomass after cellulose and comprisesabout 25% of land based biomass. Lignin is a complex heavy molecular weight, heterogeneous aromatic polymer (Maruthamalai Rasi et al., 2014).If lignin is not recycled in nature leading to causes a serious pollution and toxicity problem in soil and aquatic ecosystem. There several factors that limit the digestibility of lignin to value added end products. The bioconversion of these materials becomes an immediate necessity to restrict ecosystem pollution (V. Sasikumar et al 2014). However, several authors reported large numbers of microorganisms from rainforest soil are capable of degrading complex lignin polymers. There are several bacterial flora has the capability to degrade lignine in to potentially beneficial end products which includes Bacillus sp., Cellumonas sp., Achromonas sp.,

Pseudomonas sp., Flavobacterium sp., Enterobacter sp., Klebsiella sp., Xanthomonas sp., Nitrobacter sp., Nitromonas sp (Harith et al. 2014). The lignin degrading bacteria has intrinsic ability to sustain in harsh environmental conditions over the fungal degradation (Shamseldin et al. 2015). In view of this, soils of Raigad district (Maharashtra, India) are selected to study biodiversity of lignocelluloses degrading bacteria. The rationale behind choosing the Raigad district area was its large forest area and huge biodiversity. In the present work we mainly focus on the isolation and identification of novel bacterial strains that has the ability to bioconversion of lignin to value added end products.

2. Materials and Methods:

Sources of isolates

The lignolytic bacteria were isolated from soils of Raigad district (Maharashtra, India). Four types of soil samples were collected from different regions of Raigad district based onforest area, rice field, coastal area, and color of soil. These soil samples were used for the isolation of lignolytic bacteria.

Alkali lignin preparation

Woody plants are the major source of lignin. The dried plant bark is collected for preparation of alkali lignin. The collected bark is dried and grinded into powder(Howard et al., 2003). The alkali lignin was prepared according to Bholy et al. (2012) 10 g of powdered bark (lignin source) added with 5ml of 1% sulfuric acid and heated in hot air oven at 80°C for 20 minutes. After cooling added with 100 ml of 4% sodium hydroxide solution and boil the mixture for 30 minutes. The dark brown colored alkali lignin was filtered and autoclaved at 121°C for 10 minutes.

Enrichment medium for lignin degrading bacteria

The enrichment medium used for enrichment of lignin degrading bacteria contains 1% alkaline lignin as sole carbon source and minimal salt medium contains (g/l of deionized water), K_2HPO_4, 4.55; KH_2PO_4, 0.53; $MgSO_4$, 0.5; NH_4NO_3, (Chandra et al., 2008) as shown in figure 5.

Enrichment of lignin degrading bacteria

The enrichment of lignin degrading bacteria from soil was done in 250 ml conical flask. 5 gm of soil sample was inoculated in 100 ml of Minimal salt medium containing 1% alkaline lignin as carbon source. The inoculated flasks were incubated on 100-120 rpm at 30-35°C temperature for 7 days.

Isolation of lignin degrading bacteria

1 mlenriched sample was transferred to 99 ml of sterile 0.9% sodium chloride (NaCl). The solution were mixed well and allowed to settle for a while. By using 1 ml of liquid mixture serial dilution were prepared in 0.9% NaCl. About 100 µl of diluted samples were spread on plate containing Minimal salt agar containing 1% alkaline lignin. The

plates were incubated at 30-35°C for 7 days until isolated colonies were developed (Rahman et al., 2013) as shown in figure 6. Morphological and biochemical characterization was done as shown in table 1.

Lignolytic activity

The bacterial isolates obtained on Minimal salt medium containing 1% alkali lignin were further screened for its lignolytic activity by using methylene blue dye as an indicator. The bacteria having lignolytic enzymes undergo oxidation of methylene blue indicator dye. For this enzyme activity assessment the isolated bacteria were streaked on LB agar plate containing methylene blue indicator (0.25gm/l). The plates were incubated at 30-35°C for 3 days. The agar plates were daily monitored for growth and decolorization of methylene blue dye as shown in figure 7. (Bondounas et al., 2011). The decolorized colonies were further processed for colony characterization. The predominant methylene blue decolorizers were *Bacillus* sp. and *Pseudomonas* sp. as shown in table 2.

3. Result and discussion:

Sources of lignolytic isolates

The lignolytic bacterial isolates were isolated from soils of Raigad district of Maharashtra India. Figure 1, Figure 2, Figure 3 and Figure 4 represents different soil samples.

Figure 1: Coastal area soil Figure 2: Forest area soil

Figure 3: Rice field soil Figure 4: Plain surface soil

Alkali lignin preparation and isolation of lignin degrading isolate

The alkali lignin was prepared according to Bholy et al. (2012). The dark brown colored alkali lignin was filtered and autoclaved at 121°C for 10 minutes. The enrichment of soil samples were done in MSM-Lignin medium as shown in Fig. 5.

Figure 5: Enrichment medium for lignin degradation

After enrichment serially diluted samples were spread on plate containing Minimal salt agar containing 1% alkaline lignin and incubated at 30-35°C for 7 days. After incubation, lignin degrading colonies showing zone on methylene blue containing medium were selected as lignin degrading isolates shown in figure 6 and 7. Bondounas et al., 2011 extracted lignin from plant bark and further used for isolation of lignin degrading bacteria from soil. This alkali lignin is used as sole source of carbon in minimal salt medium. The dark brown colored alkali lignin was stored in tight glass container and used for further experiments.

Figure 6: Plates showing growth of lignin degrading bacteria

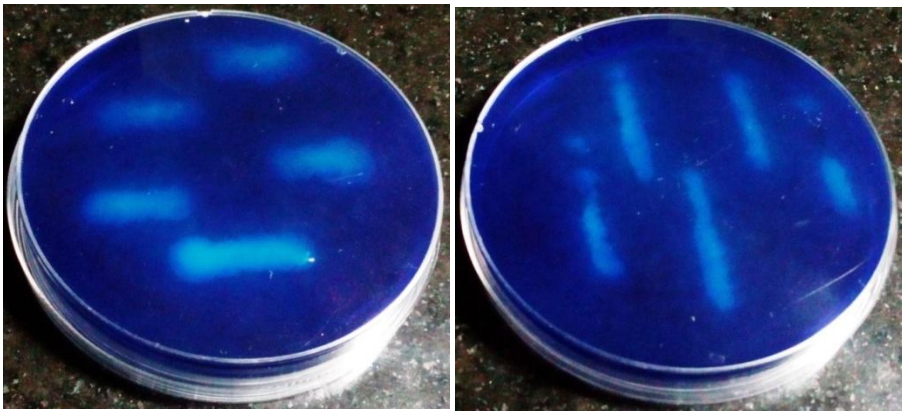

Figure 7: Plates showing lignolytic activity of bacteria

Many researchers reported the same methodologies for isolation of lignin degrading microorganisms [REF].

Based on morphological and biochemical characterization a total 14 lignolytic isolates were obtained from minimal salt medium containing 1% alkali lignin containing soils of Raigad district and their lignolytic activity was checked. The lignolytic bacteria showed clear zones on the plate's minimal salt medium containing 1 % lignin. (Sasikumar et al., 2014) . Based on the results indicated in Table 1 and Table 2 the preliminary predominant isolates were identified as *Bacillus* sp. and *Pseudomonas* sp. The isolates were then maintained on nutrient agar medium for further studies.

Table 1: Bacterial colonies were isolated on minimal salt agar medium containing 1% alkali lignin.

Sr. No.	Name of Isolate	Colony characters						Gram's nature
		Size	Shape	opacity	color	margin	elevation	
1	LD-01	1-2 mm	round	opaque	Off white	entire	raised	Gram +ve long rod
2	LD-02	2 mm	round	opaque	Off white	entire	raised	Gram +ve short rod
3	LD-03	1-2 mm	round	opaque	Off white	entire	raised	Gram +ve cocci
4	LD-04	1-2 mm	round	opaque	Off white	entire	raised	Gram +ve long rod
5	LD-05	1 mm	round	opaque	Off white	entire	raised	Gram +ve short rod
6	LD-06	1-2 mm	round	opaque	Off white	entire	raised	Gram +ve short rod
7	LD-07	1-2 mm	round	opaque	Off white	entire	raised	Gram +ve rod
8	LD-08	1-2 mm	round	opaque	Off white	entire	raised	Gram -ve rod
9	LD-09	1-2 mm	round	opaque	Off white	entire	raised	Gram +vecocci
10	LD-10	1 mm	round	opaque	Off white	entire	raised	Gram +vecocci
11	LD-11	1-2 mm	round	opaque	white	entire	raised	Gram +vecocci
12	LD-12	1-2 mm	round	opaque	Off white	entire	raised	Gram +ve rods
13	LD-13	1-2 mm	round	opaque	Off white	entire	raised	Gram +vecocci
14	LD-14	1-2 mm	round	opaque	Pale yellow	entire	raised	Gram -ve rod

Table 2: The biochemical method of identification of bacteria is a classical method of characterization of bacteria. The above isolated and morphologically characterized bacterial colonies used for biochemical tests and observations were tabulated below

Sr. No.	Name of Isolate	Formation of Endospore	Motility	Biochemical Tests						Methylene blue dye oxidation	Tentatively Identified Bacteria
				Catalase	Oxidase	IMViC Tests					
						Indole	MR	VP	Citrate		
1	LD-01	+	-	+	-	-	-	+	-	+	*Bacillus* sp.
2	LD-02	+	+	+	-	-	-	-	-	+	*Bacillus* sp.
3	LD-03	-	-	+	-	-	-	-	-	-	*Micrococcus* sp.
4	LD-04	+	+	+	-	-	-	+	-	+	*Bacillus* sp.
5	LD-05	+	-	+	-	-	-	+	-	+	*Bacillus* sp.
6	LD-06	+	-	+	-	-	-	+	-	+	*Bacillus* sp.
7	LD-07	+	+	+	-	-	-	-	-	+	*Bacillus* sp.
8	LD-08	-	+	+	+	-	-	+	+	+	*Pseudomonas* sp.
9	LD-09	-	-	+	-	-	-	-	-	-	*Micrococcus* sp.
10	LD-10	-	-	+	-	-	-	-	-	-	*Micrococcus* sp.
11	LD-11	-	-	+	-	-	-	-	-	-	*Micrococcus* sp.
12	LD-12	+	+	+	-	-	-	-	-	+	*Bacillus* sp.
13	LD-13	-	-	+	-	-	-	-	-	-	*Micrococcus* sp.
14	LD-14	-	+	+	+	-	-	-	+	+	*Pseudomonas* sp.

However, Shashikumar et al., 2014 reported almost similar results while analyzing cow dung, soil samples, and paper pulp effluent. The study of Harith et al., 2014 also reported *Bacillus* sp. isolated from decayed plant having ability to degrade lignin more effectively.

4. Conclusion:

Lignolytic bacteria were isolated from soil samples. The studied soil samples from Raigad district contains bacteria like *Bacillus* sp. and *Pseudomonas* sp have the potentials of degrading lignocellulytic wastes. These strains are capable of degrading the biomass containing lignin. Further, a complete microbial identification, optimization and characterization work is required to improve the lignin degradation of the lignocellulytic waste materials.

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