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RESEARCH ARTICLE

Isolation and Preliminary Screening of Lignin Degrading Microbes

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Abstract

Isolation of lignin degrading microorganisms from cow dung, soil compost, and paper pulp effluent was carried out in the present investigation. Totally 9 isolates of lignin degrading microbes were isolated using minimal salt media containing lignin (MSM-L) and lignolytic activities were preliminary screened by testing against methylene blue indicator dye containing LB medium. Eight microbes showed positive results and the predominant isolates were identified as *Pseudomonas* sp. The findings indicated that *Pseudomonas* sp. has significant potential for use in the applications for the treatment of lignin degradation and lignin related environment pollutants.

Keywords: Lignin, lignin degrading microbes, lignolytic activity, MSM-L medium, *Pseudomonas* sp.

Introduction

Lignin is the most structurally complex carbohydrate possessing a high molecular weight and the most recalcitrant, consisting of various biologically stable linkages (Perez *et al.,* 2001). The lignocellulose material of plant consists of three main compounds, namely cellulose, hemicellulose and lignin. After cellulose, lignin is the second most abundant renewable biopolymer in nature. It is most abundant aromatic polymer in the biosphere (Rahman *et al.,* 2013). Lignin causes a serious pollution and toxicity problem in aquatic ecosystem owing to its low biodegradability. Large amounts of lignocellulosic waste generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agro-industries pose environmental pollution problems (Howard *et al.,* 2003). In soil biosphere, a few groups of organisms are capable of degrading complex lignin polymers and best examples are white rot fungi and other fungi such as *Phanerochaete chrysoporium, Streptomyces viridosporus, Pleurotus eryngii, Trametes trogii, Fusarium proliferatem, Agaricus, Erwenia, Copricus, Mycema* and *Swterium* (Alexender, 1977). However, commercialization of lignin degradation by fungi has disadvantages in the form of problems related to fungal protein expression and genetic manipulations and showed a lack of stability under practical treatment condition involving high pH, oxygen limitation and high lignin concentrations (Crawford and Muralidhara, 2004). For this reason, studies on the bacterial degradation were more preferable for lignin and the production of bacterial ligninolytic enzymes has seen increased in recent year (Renugadevi *et al.,* 2011). 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.

Lignin peroxidase (LiP) is an enzyme first discovered in 1983 was used to degrade lignin. Lignin peroxidase is useful in the treatment of colored industrial effluents and other xenobiotic as it has bioremediation potential to decolorize the effluents (Shi *et al.,* 2013). Decolorization of methylene blue dye was also used as an indicator of the oxidation ability of ligninolytic enzyme produced by the potential lignin degrading bacterial strains (Rahman *et al.,* 2013). Lignocellulytic enzymes also have significant potential application in various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper, and agriculture (Howard *et al.,* 2003). In this study, an investigation was attempted to prepare alkali lignin substrate and isolation, degradation and characterization of lignin degrading bacteria were made feasible.

Materials and methods

Alkali lignin preparation: Lignocellulose is the major structural component of woody plants and non-woody plants such as grasses and represents a major source of renewable organic matter. The plant sources used for extraction of lignin were dried and bark grinded to powder (Fig. 1) (Howard *et al.,* 2003). With 10 g of powdered bark (lignin sources), 5 mL of 1% sulfuric acid was added and heated in hot air oven at 80°C for 20 min and allowed to cool followed by 100 mL of 4% sodium hydroxide and boiled for 30 min. The dark brown colored alkali lignin was filtered and autoclaved at 15 lbs for 10 min (Bholy *et al.,* 2012).

Isolation of lignin degrading bacteria: The sample sources used for the isolation of lignin degrading bacteria were from cow dung, plant composing soil and paper pulp effluent from Erode district (Fig. 2).

Fig. 1. Preparation of lignin sources
a) Dried plant barks, b) Powdered bark.

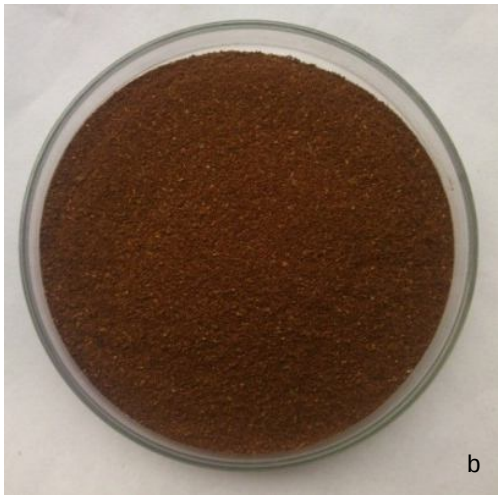


Fig. 2. Sample source a) Cow dung, b) Plant composting soil.

Fig. 3. Enriched medium cultured with lignin degrading microorganisms.

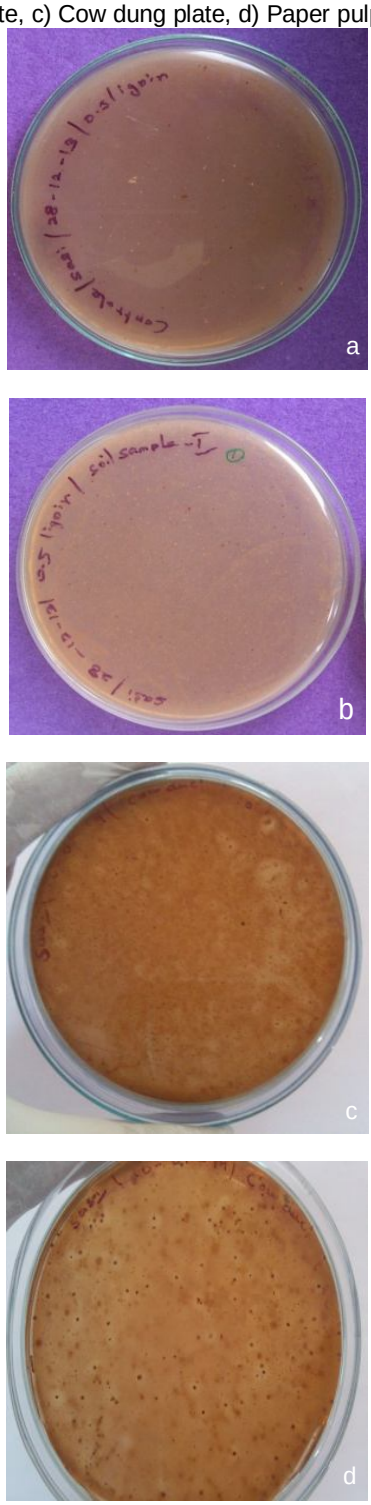
The sample was collected in a sterilize polythene bag and transported to the laboratory and stored at -20°C. The lignin degrading bacteria was enriched using a medium in which lignin provided the sole carbon and energy source (MSM-L). MSM-L consisted of 1% alkaline lignin minimum salt medium solution which contained (g/L of deionized water) K_2HP0_4, 4.55; KH_2PO_4, 0.53; $MgSO_4$, 0.5; NH_4NO_3, 5 (Chandra *et al*., 2008). The components were mixed and the resulting suspension was autoclaved. Enrichment culture were performed in 250 mL Erlenmeyer flask by placing 5 g sample in 95 mL MSM-L and culture were incubated at 120 rpm for 7 d at 30 to 45°C (Fig. 3). Enriched sample of 1 mL were transferred to 99 mL of sterile 0.9% NaCl. The solution were stirred vigorously and allowed to settle down. Using 1 mL of the liquid mixture, serial dilution technique was performed from each dilution. About 100 µL of serially diluted sample were spread on plate containing minimal salt medium agar containing alkaline lignin. The plates were incubated at 30°C for 7 d until colonies developed. The isolated bacteria were plated onto fresh MSM-L agar plates repeatedly to obtain pure cultures (Rahman *et al*., 2013).

Lignolytic activity: The bacterial isolates were further screened using methylene blue dye as an indicator. The microbes possess lignolytic enzymes undergoes oxidation of indicator dye. The isolated bacteria were streaked on methylene blue indicator dye (0.25 g/L) containing LB agar plate. The plates were incubated at 30°C for 72 h. The agar plates were monitored daily for bacterial growth and decolorization of the methylene blue dyes (Bondounas *et al.,* 2011). The decolorized microbial colonies were processed for identification.

Results and discussion

The crude lignin was extracted from natural sources to obtain maximum lignin degrading microbes. The extraction was done by alkaline delignification method using 4% sodium hydroxide, a dark color alkali lignin was obtained which was stored in an air tight bottle and used for further experiments.

Fig. 4. Degradation of lignin plates a) Control plate, b) Compost soil plate, c) Cow dung plate, d) Paper pulp plate.



Totally 9 lignolytic degrading isolates were obtained from the MSM-L plate containing compost soil, cow dung and paper pulp effluent and their lignolytic activity was checked. The lignolytic microbes showed clear zone of lignin degradation in each plate shown in Fig. 4. The isolates were further acclimatized to higher concentration from 1% lignin for 7 d and maintained as pure culture in MSM-L plates shown in Fig. 5.

Fig. 5. Isolated pure culture of lignin degrading bacteria using streak plate method.

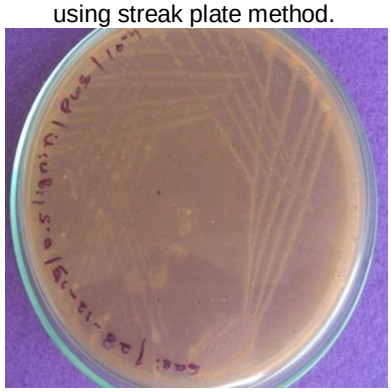
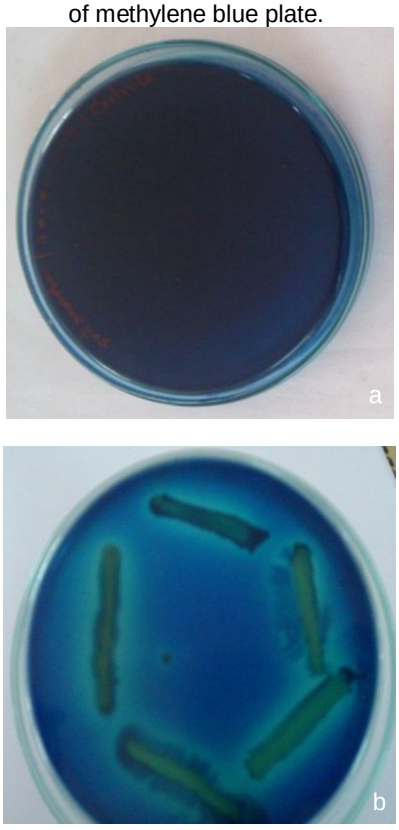


Fig. 6. Lignolytic activity plate a) Control plate, b) Decolorization of methylene blue plate.



Screening of lignolytic microorganisms were done using MSM agar containing lignin (L) added directly to the MSM agar medium as the sole carbon source. About 9 bacterial strains were obtained and further screening was carried out by platting the isolates in a new MSM-KL medium containing methylene blue as the lignin polymeric dye and incubated for 7 d or LB with methylene blue (25 mg/L) plate incubated for 72 h (Fig. 6). The decolorization of methylene blue has been used previously as an indicator of lignin peroxidase enzyme activity (Ferreira-leitao *et al.,* 2007). Changes in color of the medium from blue to clear were observed for 8 bacterial strains. The predominant methylene blue decolorizers were *Pseudomonas* sp. upon characterization using standard protocol.

Conclusion

This little piece of investigation is only a preliminary step to isolate lignin degrading microorganisms. The findings indicate that *Pseudomonas* sp. has significant potential use in the applications for the treatment of lignin degradation and lignin related environment pollutants.

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