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International Journal of Current Research
Vol. 10, Issue, 06, pp.70424-70429, June, 2018

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

ISOLATION AND IDENTIFICATION OF LIGNINOLYTIC MICROBES

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ARTICLE INFO

Article History:
Received 08th March, 2018
Received in revised form
22nd April, 2018
Accepted 29th May, 2018
Published online 30th June, 2018

Key words:

Lignin, ligninolytic microbes, MSML, methylene blue, Nutrient Agar.

Abbreviations:

BOD- Biological Oxygen Demand
IMViC- Indole Methyl Red Voges-Proskauer Citrate
LiP- Lignin Peroxidase
LMWAC- Low Molecular Weight Aromatic Compounds
MnP- Manganese peroxidase
MSML- Minimal Salt Media containing Lignin
NB- Nutrient Broth

ABSTRACT

Background: Lignin is an organic polymer made up of various aromatic compounds which in combination with cellulose and hemicellulose forms a chief partof the woody tissues. Wood processing industries like paper-pulp industries have been among the most significant of industrial polluters of the waterways and environment. The pulp and paper industry produces large quantities of toxic brown/black effluent (around 100 million kg) that comes primarily from lignin and its derivatives (e.g., lignosulphonic acid, resins, phenols, and hydrocarbons) that are released during various processing steps of lignocellulosic materials. Discharge of such untreated effluent results in increased BOD, slime growth, thermal problems, scum formation, discoloration, loss of aesthetic quality and toxicity to the aquatic life, in the receiving water bodies. These problems can be overcome by biological treatment using certain ligninolytic microbes processing ligninolytic enzyme systems.
Objective: The goal of this research work was to isolateligninolytic microbes from various environmental niche and to screen them for potential ligninolytic activity.
Methods: The lignin extract was prepared from saw dust. The ligninolytic bacteria were isolated from samples of garden soil, sewage and compost using Minimal Salt Medium containing lignin extract(MSML). The isolates were qualitatively screened for ligninolytic activity using Methylene blue dye reduction test. A comparative study of growth rates of screened isolates in MSML and Nutrient broth (NB) was performed. Various biochemical tests were conducted for identification of screened isolates.
Result: Ligninolytic microbes were isolated from various environmental niche. The isolated microbes were screened based on their potential to reduce the basic dye methylene blue. The isolates showed varied growth rates in MSML and NB with few having more affinity to lignin. The isolates were found to be Gram positive bacilli and cocci along with few being actinomycetes. Few isolates were motile showing varied biochemical characteristics.
Conclusion: Ligninolytic microbes were found in garden soil, sewage and compost samples.The dye reducing activity of the isolates indicates the presence of the oxidative ligninolytic enzymes. Isolates showing high growth rate in MSML had greater efficiency of lignin degradation.

Citation: Madhumita Ghosh Dastidar, Akshaya Simha, N., Ananth Koushik, B., Aradhya, D., Harishmitha, N., Nayana, S., Nidhish, P., Shakthi Vignesh, S., Siri, G. and Tavisha, V. K., 2018. "Isolation and identification of ligninolytic microbes", *International Journal of Current Research*, 10, (6), 70424-70429.

INTRODUCTION

Lignin is a complex organic polymer which along with hemicellulose and cellulose forms the cell wall of woody tissues. It is composed of three principal building blocks: p-coumaryl alcohol (p-hydroxyphenyl propanol), coniferyl alcohol (guaiacyl propanol), and sinapyl alcohol (syringyl propanol) which is bound by various ether and carbon-carbon bonds. (Lebo *et al*., 2001). These units occur in different ratios in different types of plants. Lignin is removed as an effluent from paper-pulp industries as it gives a coarse texture and brown color to the paper (Gonzalo de Gonzalo *et al*., 2013).

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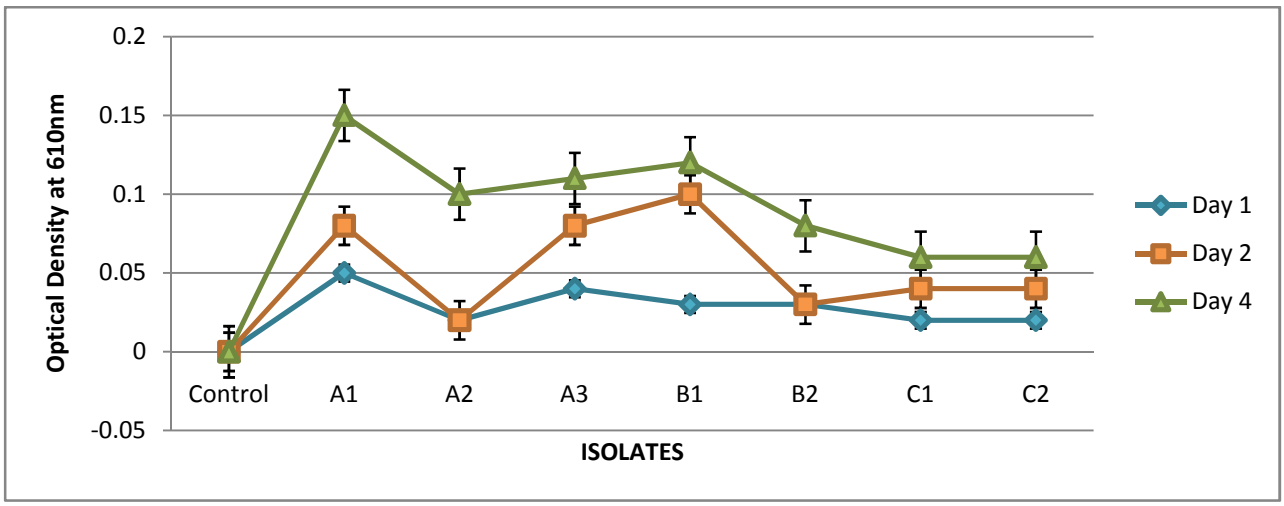
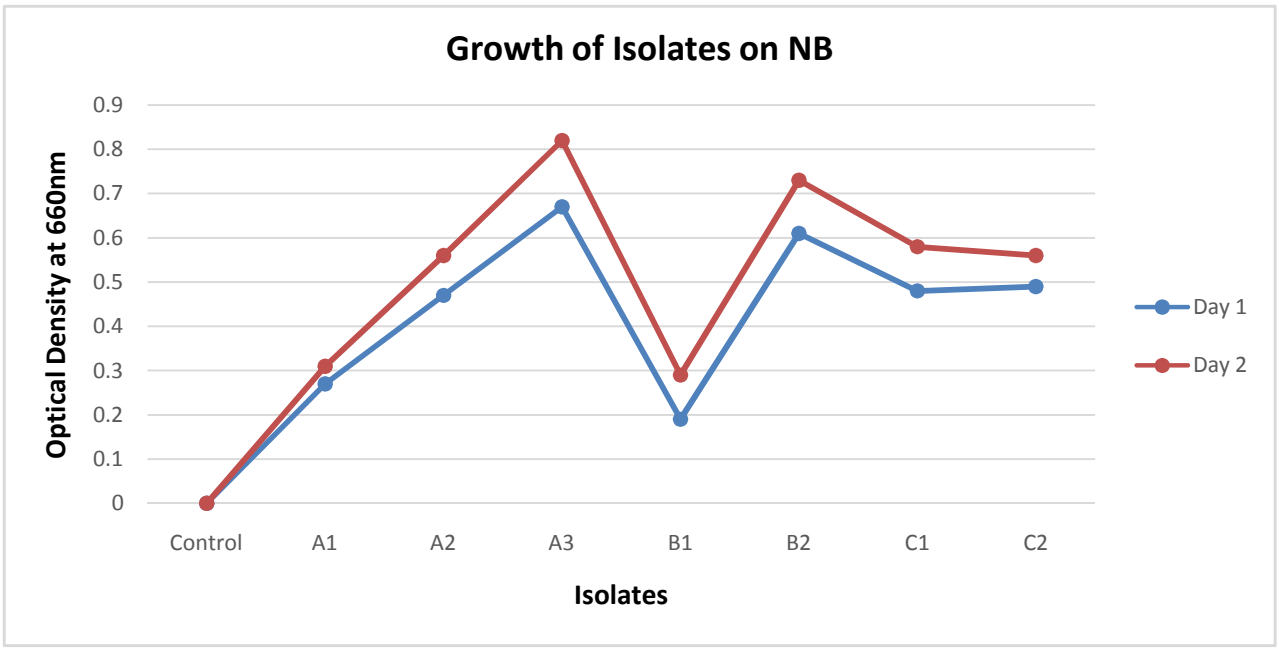
DOI: https://doi.org/10.24941/ijcr.31186.06.2018

The removal process releases dark colored effluents containing components of lignin and its derivatives. These are toxic when released into the water bodies, which creates a need to degrade lignin in a more natural way. The idea of degrading lignin polymer can be biologically achieved through microbial lignin degradation by the production of ligninolytic enzymes produced by specific ligninolytic fungi and bacteria (Rahman, *et al*., 2013). The depolymerization is caused by some ligninolytic enzymes such as Lignin peroxidase (LiP), Manganese peroxidase (MnP) and laccase secreted by some microbes. The efficiency oflignin degradation may depend on plant species and types of tissues(Howard *et al*., 2003). The ligninolytic activity of fungi has been greatly studied than bacteria, but recently many degrading enzymes of bacterial sources has been analyzed (Bugg *et al.,* 2011b). Certain fungi like White rot fungi, Brown rot fungi and bacteria like alpha



Graph 1. Growth of ligninolytic microbes on MSML



Graph 2. Growth of ligninolytic microbes in Nutrient broth

proteo-bacteria, Actinomycetes is actively involved in biodegradation of lignin. However, commercialization of lignin degradation by fungi has disadvantages in the form of problems related to fungal protein expression and genetic manipulations and shows a lack of stability under practical treatment conditions involving high pH, oxygen limitation and high lignin concentrations (Crawford and Muralidhara, 2004). For this reason, studies on the bacterial degradation are more preferable for lignin degradation and the production of bacterial ligninolyticenzymes (Renugadevi *et al*., 2011). In this study, an investigation was attempted to isolate ligninolytic bacteria from various environmental niche using minimal media containing alkaline lignin extract. The isolates were identified, screened and assayed for ligninolytic activities.

MATERIALS AND METHODS

Lignin extract preparation

Saw dust was used as a source of lignin. 10g of saw dust was treated with 5ml of 1% Con.H_2SO_4. This mixture was then heated in hot air oven for 20mins at 80°C.

After the stipulated time, the mixture was cooled and boiled with 100ml of 4% NaOH solution as solvent. Dark brown colored solution obtained was filtered and the extract was used for further experiments. (Bholy *et al*., 2012)

Isolation of ligninolytic microbes

Ligninolytic microorganisms were isolated from garden soil, sewage and compost. 10% of each sample in saline (0.9% NaCl) was serially diluted up to 10^{-2}(1:100). 1ml of eachdiluted samples along with approximately 15ml of molten Minimal Salt Media containing 1% lignin extract (MSML) were mixed in petri plates and were incubated at 37°C for 7 days. MSML consists of potassium dihydrogen phosphate(3g), disodium hydrogen phosphate(6g), sodium chloride(5g), ammonium chloride(2g), magnesium sulphate(0.1g), agar-agar(15g)in 1000ml of distilled water (pH 7). 1% lignin extract as sole carbon source was added to the sterilized medium. (Chandra R *et al*., 2008). MSML appears brown due to the presence of lignin in it. Ligninolytic microbes use this lignin and a characteristic decolorization is observed around the colonies. These colonies were further sub-cultured many times to obtain pure culture.

Table 1. Result of Biochemical tests

Isolates	*A1*	*A2*	*A3*	*B1*	*B2*	*C1*	*C2*
Gram staining reaction	Actinomycetes	Actinomycetes	Gram +ve Bacilli	Gram +ve Bacilli	Gram +ve Cocci	Gram +ve Bacilli	Actinomycetes
Motility	--	--	+ve	+ve	-ve	+ve	--
Results of carbohydrate fermentation							
Glucose fermentation *Acid*	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Gas	+ve	-ve	-ve	+ve	+ve	-ve	-ve
Lactose fermentation *Acid*	-ve	-ve	+ve	+ve	-ve	-ve	-ve
Gas	-ve	-ve	-ve	+ve	-ve	-ve	-ve
Sucrose fermentation *Acid*	-ve	+ve	+ve	+ve	-ve	-ve	-ve
Gas	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Results of IMViC test							
Indole production	-ve	-ve	-ve	-ve	+ve	+ve	-ve
Methyl red test	-ve	-ve	-ve	+ve	-ve	-ve	+ve
VogesProskauer test	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Citrate utilization test	-ve	+ve	+ve	+ve	+ve	+ve	+ve
Results of other biochemical tests							
Starch hydrolysis	-ve	+ve	+ve	-ve	+ve	-ve	+ve
Gelatin liquefaction	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Catalase test	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Oxidase test	+ve	+ve	+ve	+ve	+ve	+ve	+ve

*A1,A2,A3 are soil isolates; **B1,B2 are sewage isolates; ***C1,C2 are compost isolates

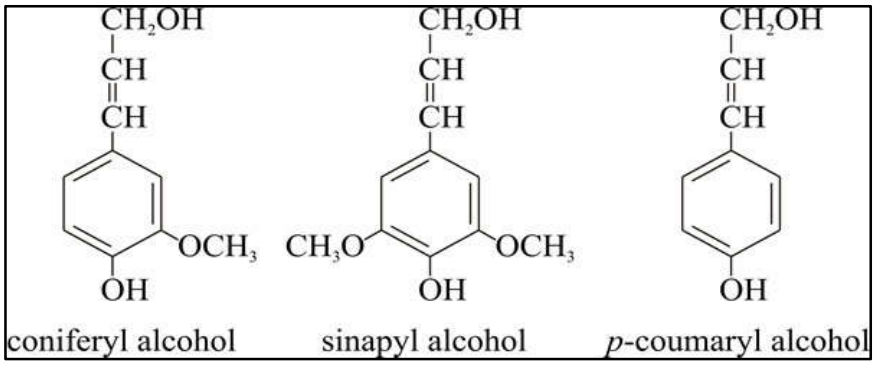


Figure 1. Subunits of Lignin. Chemical Structures of Phenylpropanoid alcohols used to construct the lignin polymer (Moore *et al.*, 2011)

Figure 2. Lignin Extract. It is the source of lignin which could be utilized by microorganisms which are lignin degraders. This is used as carbon source for MSML Medium

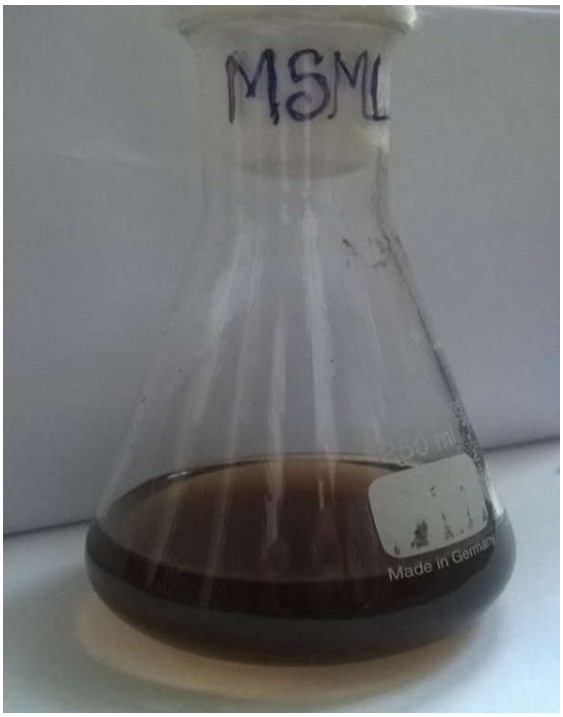


Figure 3. Minimal salt media. Preparation of Enrichment Media by adding 1% of lignin extract and soil samples for isolation of ligninolytic microorganisms

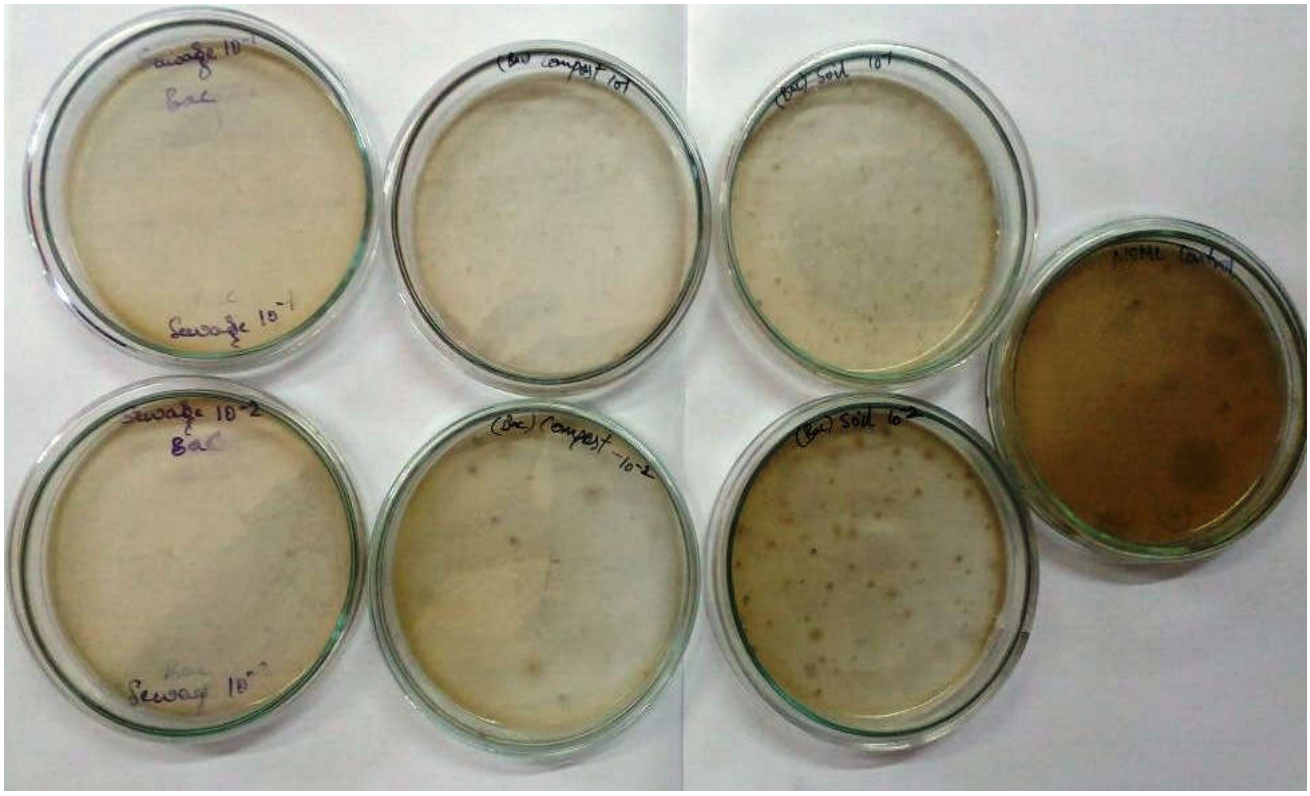


Figure 4. Isolation of lignin degrading bacteria in MSM enriched medium. The conversion from dark brown colour of medium to colourless indicated growth of ligninolytic bacteria

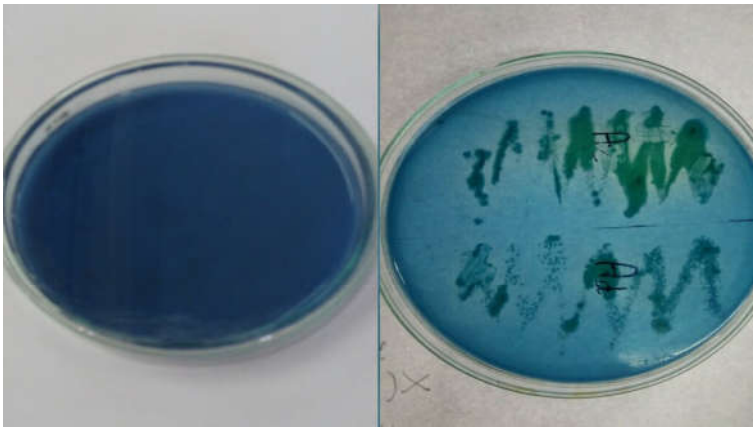


Figure 5. Dye decolorization test for methylene blue by using stock concentration of dye of 25mg/L. and incubated the plate for 48 hrs after inoculating with lignin degrading isolates. Colour changes observed only in experimental plate indicated the presence of lignin degrading isolates

Screening of ligninolytic microbes: The isolates obtained in MSML media were screened qualitatively using Methylene blue dye reduction test. MSML agar plates containing methylene blue dye was prepared and isolates were streaked on it. These plates were incubated at 37℃ for 7days. The reduction of basic dyes like methylene blue indicates the presence of oxidative ligninolytic enzymes. The clear zone around the colonies indicates the microorganism are likely to be lignin degraders(Bondounas *et al*., 2011). The screened isolates were simultaneously grown in nutrient broth and MSML broth. The growth rate of each isolate was estimated spectrophotometrically at 600nm.It is significant in identifying potent and/or sole lignin degraders (Huang *et al* 2013).

Identification of ligninolytic microbes: Isolates obtained from MSML media were identified by the following bio-chemical tests:

Gram-staining, motility test, fermentation test, starch hydrolysis, gelatin-liquefaction test, IMViC test, catalase and oxidase test (Aneja 1999).

RESULTS

Lignin extract was prepared from saw dust using sodium hydroxide as solvent. Bacterial colonies appeared on MSML agar plates after 7 days of incubation at 37℃ from samples of garden soil, sewage and compost. Totally 4 isolates from garden soil, 7 isolates from sewage and 2 isolates from compost were obtained in MSML. These isolates were screened for dye reducing ability using methylene blue dye. Few isolates showed clear zones in the dye plate indicating the presence of ligninolytic enzymes. After screening, 3 isolates from soil (A1, A2, A3), 2 isolates from sewage (B1, B2) and 2 isolates from compost (C1, C2) were found to show significant

ligninolytic activity. Each of these isolates when grown in media having different primary carbon source i.e., MSML (lignin) and NB(glucose), showed different growth rates. The growth rate in the above said media is as follows:

The growth of ligninolytic bacteria in MSML broth was measured quantitatively. The Optical Density of the cultures was recorded at 600nm. Growth of isolates from compost, which is C1 and C2 was seen lesser than the others. The isolates A1, B1 and A3 have shown higher growth. This confirms the presence of ligninolytic enzymes in A1, B1 and A3 isolates. The growth of the isolates from compost i.e., C1 and C2 has shown slightly more growth in NB than in MSML. The soil isolates A2, A3 and the sewage isolate B2 has shown similar growth in both MSML and NB. A1 and B2 have shown significantly lesser growth in NB than MSML. These screened isolates were subjected to various biochemical tests as a part of identification. The result of these biochemical tests are tabulated in the Table 1.

DISCUSSION

Lignin degradation provides various compounds like cellulose etc. having wide applications making it a necessary process. Biological degradation of lignin finds greater applications in modern industries over traditional methods. Several microorganisms like bacteria and fungi are known to degrade lignin by its enzymes. Lignin degradation is widely studied in fungi species belonging to ascomycetes, e.g., *Trichodermareesei*, basidiomycetes, e.g., white rot-*Phanerochaetechrysosporium* and brown rot-*Fomitopsispalustris* (Mehdi Dashtban *et al*., 2010). Besides these fungi, there are reports of bacteria that have the ability to break down lignin (Bugg *et al*., 2011a; Zimmermann, 1990). The lignin degrading bacteria isolated from soil are Actinomycetes, α-proteobacteria, and γ-proteobacteria (Bugg *et al*., 2011a). By the mid-1980s, developments in the understanding of bacterial lignin degradation mechanisms were obtained from Actinomycetes and Pseudomonas species. The bacteria which degrade lignin (Masai, 2002) include Actinomycetes such as Nocardia, Rhodococcus, *Sphingomonaspaucimobilis* SYK-6 and *Streptomyces viridosporus* T7A which, when grown on lignocellulose, produces extracellular peroxidases that degrade both the lignin and carbohydrate components of lignocellulose (Saha *et al*., 2017). In comparison to fungal lignin degrading enzymes, the bacterial enzymes implicated in lignin breakdown are much less studied. It was suggested that bacteria might release extracellular ligninolytic enzymes (McLeod *et al*., 2006). In this study, the lignin degrading bacteria were isolated from the garden soil, sewage and compost. The types of samples selected were thought tosupport lignocellulosic and lignin-like compounds and therefore microbes in that niche have more enzymes to degrade lignin. Gram reaction showed that soil samples contain Actinomycetes and Gram positive bacilli, sewage contain both Gram positive and Gram negative bacteria and compost showed the presence of positive bacilli and Actinomycetes. Use of lignin-related LMWACs by bacteria as a sole carbon source has been taken as criteria for selection of ligninolytic bacterial strains (Kato *et al*., 1998). The aerobic degradation of aromatic hydrocarbons by microorganisms has been investigated extensively (Ferhan *et al*., 2013). In addition, microorganisms, that can degrade aromatic compounds anaerobically by a reductive enzyme system, have been reported (Bugg *et al.,* 2011b). In this study the potential bacteria with ligninolytic capabilities are A1, A3 and B1 isolates because these bacteria showed higher growth when challenged in MSML medium where lignin as sole carbon source. These bacteria grow well when glucose is used as co-substrate (Sing *et al.,* 2013). Chandra *et al.,* (2011) reported that lignin degrading bacteria need glucose as co-substrate to aid in the degradation of lignin. Therefore in lignin degrading experiment, glucose was added with in the medium as co substrate to support bacterial growth to facilitate the lignin degradation. It was observed that the soil isolates A2, A3 and B2 has shown similar growth in both MSML and NB. A1 and B2 have shown significantly lesser growth in NB than MSML. C1 and C2 has shown slightly more growth in NB than in MSML. The ligninolytic enzymes also have a tendency to degrade certain basic dyes. Here Methylene blue dye was taken into consideration to check dye decolorization. Hence, it acts as an indicator for enzyme activity. All the isolates showed dye reduction, especially A1, A3, B1 showed rapid reduction of methylene blue compare to other isolates. The decolorization of methylene blue can be correlated to lignin degrading enzymes released by isolated bacteria. The white rot fungus *Phanerochaetechrysosporium* was already reported to decolourise azo dyes in 1990. Since then a number of reports studying the ability of different white rot fungi for decolouration of various dyes have been increasing (Xionng *et al*., 2013). Some actinomycetes and anaerobic bacteria also have dye degrading activity, possessing lignin degrading enzymes (Arora *et al*., 2002). Efforts to isolate bacterial cultures capable of degrading azo dyes started in the 1970s with reports of Bacillus subtilis (Sasikumar *et al*., 2012), *Aeromonashydrophila* (Tomonori *et al*., 2002) followed by Bacillus cereus (Stuart *et al*., 2001). Bioremediation of Lignin plays an important role. Bioremediation is removal of unwanted organic chemicals and it deals with the anthropogenic substances, present in nature, which are recalcitrant. It is cost effective. It replaces physical and chemical means of degradation of pollutants. The role of microorganisms and its enzymes in bioremediation especially with lignin is established.

Conclusion

- The ligninolytic bacteria were isolated from various natural nichelike soil, sewage and compost.
- These isolates were able to reduce the basic dye and were proved to be potent lignin degraders.
- The comparative study of growth rate in different media showed that the isolates were able to utilize lignin as its sole carbon source.

Acknowledgement: The authors pay thanks to Microbiology Department of Vijaya College to carry the project.

Conflict of Interest: The Authors Declare That There Is No Conflict of Interest.

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